

UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE BIOLOGIA ANIMAL



**From Plasticity to Robustness:
Mechanisms coordinating organ size and pattern**

André Nogueira Alves

Mestrado em Biologia Evolutiva e do Desenvolvimento

Dissertação orientada por:
Doutora Christen Mirth, School of Biological Sciences, Monash University,
Melbourne, Austrália
Doutor Élio Sucena, Faculdade de Ciências da Universidade de Lisboa,
Lisboa, Portugal

2017

Agradecimentos

Agradecer, ou noutras palavras: “Obrigado por me terem dado parte do vosso coração”. E porque, com cada pedaço de coração que fui recebendo, e por vezes mais do que isso, consegui manter as minhas forças e a minha sanidade mental ao longo do ano, agora é a minha vez de retribuir e dar parte do meu coração.

Primeiro quero agradecer a toda a comunidade da Universidade de Monash, em Melbourne, por me fazerem rir às horas de almoço ou fora delas. Por serem curiosos e quererem saber mais do meu trabalho, obrigando-me a pensar nas melhores maneiras de apresentá-lo.

Aos membros dos laboratórios de *Drosophila* do espaço de laboratório onde estive, ajudaram-me incontáveis vezes e faziam-me companhia por várias noites que lá se fez.

A todos os membros do laboratório onde estive, sem eles não perceberia quão complicado é este projecto para quem não trabalha nele. À Ansa por aturar-me na quantidade de vezes que dizia que estava esfomeado, e por ser a primeira a levantar-se e chamar-me caso houvesse comida de graça na sala comum. À Celina por ajudar-me nas minhas experiências e deixar-me atazaná-la horas sem fim. E ao Nuno pelas conversas não relacionadas com trabalho, mas sim sobre livros, séries e tantos outros assuntos. Ajudaram-me a ter uma mente crítica e sã, elemento crucial nos últimos meses.

A todos os elementos associados ao meu mestrado, o meu obrigado, o primeiro ano não seria nada sem eles e o segundo também não seria grande coisa. Obrigado aos meus colegas e aos professores por proporcionarem um mestrado brilhante.

À minha família, por apesar de ser gigantesca, lembrarem-se sempre do “André que foi para a Austrália e que um dia será o doutor da família” com um orgulho sem comparação, sem que alguma vez o tenha pedido. O que fiz nesta tese é um mistério do universo para eles, mas mesmo não sabendo, eles acreditam sempre que farei o meu melhor.

Aos seis, que comigo formam o C7ayton, Cass, Kevin, Emily, Mario, Helena e Tent. Foram todos essenciais, cada um à sua maneira, quer dando apoio, quer criticando. Por todas as vezes que apoiaram, com bolos, doces, palavras simpáticas ou apenas dois dedos de conversa com um café, um gigantesco obrigado. Por todas as vezes que criticaram com, “não consegues fazer isto”, “para de fingir que trabalhas”, ainda maior obrigado. Por vezes um não cria uma maior força de vontade do que um “sim”. Pelas vezes que me fizeram rir, como quando coseram sinos à minha bata para não os assustar, e como, mesmo com 6 sinos, conseguia assustá-los a todos. Por serem dos primeiros a perguntar se precisava de ajuda com alguma coisa, mesmo quando estavam ocupados com tudo. As minhas memórias com estes seis serão sempre recordadas com alegria.

À única Velha, Eduarda, ou Vyteer, por mesmo a milhares de quilómetros, 11 horas de diferença horária e muito mais, conseguir estar a par tudo e pronta para dar tudo de si caso fosse necessário. Nos bons e maus momentos, estava lá, nem que fosse calada, a sua presença foi suficiente em muitos momentos para manter a minha sanidade. Por continuar a ser assim e não mudar nunca. Por ser aquela pessoa que ajuda outra e fica na véspera de entregar uma tese até de madrugada acordada. Espero que esta amizade continue por muitos mais anos.

Aos meus pais, por me terem criado da maneira que sou, cada um com os seus erros, mas por me terem apoiado incondicionalmente em cada decisão que fizesse. Apesar de serem um duo estranho e impossível de se aturar juntos, as palavras que teria para vos agradecer nunca seriam suficientes.

À minha supervisora, Christen Mirth, primeiro por me ter deixado ter esta aventura, sem ela, nada disto teria sido possível. Segundo, por ter puxado por mim, sabendo que só atingindo os meus limites seria capaz de ultrapassá-los. Terceiro por me ter ajudado na escrita da tese e ficar mais stressada do que eu com a data limite de entrega, esta tese seria muito pior se não tivesse tido todo o apoio proveniente dela. Quarto, por ser a pessoa brilhante e amigável que é, uma inspiração para

muitos e um modelo do que é ser um PI como deve ser. Espero um dia poder ser como ela, e que eu seja um motivo de orgulho para ela também.

Por último, quero agradecer ao acaso, ou como outras pessoas o chamariam, destino, por ter sido aleatório da maneira certa, por ter reunido as condições ideais, e ter permitido que tudo isto acontecesse e que conhecesse todas estas pessoas.

Um muito obrigado a todos...

André N. Alves

Abstract

Environments are spatially and temporally dynamic, which poses a particular challenge to organisms as they try to tune their development to optimise their traits for a given environment. For some traits, it is beneficial to alter traits according to environmental conditions – a process known as developmental plasticity. Other traits need to maintain constant phenotypes across environmental conditions, known as developmental robustness. Even though we understand some of the mechanisms that give rise to plasticity or robustness in traits, little is known of how these processes are coordinated within organs so that these structures can exhibit both plastic and robust phenotypes. In *Drosophila melanogaster*, the wing development is well characterized and this organ displays both plasticity, in the form of wing size, and robustness, in the form of wing pattern. Previous studies in wing have shown that although the final pattern of the wing is robust, the rates of patterning in this organ show considerable variation throughout the third larval instar. Signalling via the steroid moulting hormone ecdysone is known to regulate both wing disc growth and patterning. This raises the question; how can the same signalling pathway coordinate both plastic and robust developmental responses? In this study, we genetically ablated the prothoracic gland (PGX), the gland that produces ecdysone, to explore how ecdysone concentrations affect the growth and patterning of the wing imaginal disc. By feeding PGX larvae a range of ecdysone concentrations, we determined how ecdysone, growth and patterning interact. We show that ecdysone triggers different responses in different traits. Early in the third instar, growth responds linearly to increasing ecdysone concentrations. Further, we observe significant differences between sexes in the response to ecdysone, with ecdysone affecting growth rates in fed males, but only affecting wing growth of the female wing discs when they are starved of protein. Wing disc patterning, on the other hand, shows threshold responses to ecdysone early in the third instar, and these responses do not differ between sexes. At the very end of development, ecdysone concentration does not affect disc growth rates. However, it does affect the rate of patterning for both Achaete and Senseless. These data suggest that ecdysone coordinates plastic with robust developmental processes via differences in how these processes respond to the hormone, both in nature and time.

Key-words: Plasticity, Robustness, Ecdysone, Growth, Patterning, Wing imaginal disc, *Drosophila melanogaster*

Resumo

Os ambientes são dinâmicos, quer temporalmente quer espacialmente. Devido a isto, os organismos têm de ter mecanismos que permitam ajustar-se às condições ambientais para poder sobreviver. Dependendo da característica em questão, os organismos podem alterar os seus fenótipos para melhor adaptar-se ao ambiente, habilidade conhecida como plasticidade fenotípica. Outras características podem ter mecanismos que permitam manter um fenótipo constante durante várias condições ambientais, conhecido como robustez fenotípica.

A plasticidade fenotípica foi profundamente estudada nos passados anos. Desde plantas que alteram a coloração das pétalas dependendo da composição do solo, até mamíferos como raposas, que alteram a coloração do pelo de forma a adaptar-se à estação do ano em que se apresentam. Os mecanismos moleculares que descrevem plasticidade foram extensamente estudados em *Drosophila melanogaster*. Apesar desta espécie de insetos não ter a plasticidade no que toca ao tamanho, e forma de corpo, que insetos sociais como formigas e abelhas têm, mudar o ambiente térmico ou nutricional, causa alterações no tamanho do corpo/órgão destas moscas-da-fruta. Como este inseto tem várias ferramentas genéticas disponíveis, torna-se um bom animal-modelo para estudar plasticidade.

Nesta espécie de drosofilídeo, o crescimento de órgãos e a taxa de crescimento são altamente reguladas pela cascata génica da insulina/alvo de rapamicina (Insulina/TOR). Insulina/TOR afeta também a duração do crescimento através da regulação da transição conhecida como peso crítico. A cascata génica insulina/TOR atua na glândula protorácica, a glândula responsável por produzir a hormona ecdisona. Esta hormona regula rigidamente o efeito da nutrição no crescimento de órgãos.

Robustez fenotípica é também importante para garantir que o fenótipo mantém-se igual ao longo de vários ambientes. A dominância em heterozigotos para a cor castanha dos olhos é um dos exemplos de robustez, visto que ter duas cópias ou apenas uma de um gene resulta no mesmo fenótipo. Em *D. melanogaster*, o disco genital é um bom exemplo de robustez, visto que muda pouco no seu tamanho relativamente ao resto do corpo quando submetido a gradientes nutricionais ou térmicos.

Em *D. melanogaster*, o padrão final de órgãos como a asa permanece constante ao longo de vários ambientes, e já foi demonstrado que a taxa de padronização é variável com condições ambientais. A hormona ecdisona é também importante neste processo. Isto demonstra que, apesar do padrão final ser robusto, a trajetória que leva ao estadio final tem de ser altamente plástica e afetada pela ecdisona. Como é que estes mecanismos, que conferem plasticidade e robustez a características do fenótipo, são coordenados, é algo ainda desconhecido.

Foi descrito em *D. melanogaster* o desenvolvimento do disco imaginal da asa no terceiro estadio larvar. Foi observado que, no desenvolvimento deste disco imaginal, há plasticidade no início do processo, na forma de trajetórias de padronização diferentes. Estas trajetórias são alteradas com diferenças na sinalização da hormona ecdisona ou até com diferenças ambientais, como temperatura. No entanto, existe também robustez, visto que mesmo que haja um atraso na padronização dos discos, estes assumem o estadio final de padronização no final do desenvolvimento.

Para tentar desvendar o funcionamento destes mecanismos, fizemos uso de larvas PGX. Estas larvas quando sujeitas a temperaturas superiores a 29°C, sofrem apoptose da glândula protorácica, e como consequência, são incapazes de produzir ecdisona. Administração de ecdisona na comida destas larvas resulta num resgatamento do fenótipo.

Utilizando estas larvas e comparando-as com duas linhas controlo, *>grim* e *PG>*, observamos o crescimento e a padronização dos discos imaginais das asas no início e no fim do desenvolvimento para tentar entender o funcionamento dos mecanismos que controlam plasticidade e robustez referidos

anteriormente. Observamos também o efeito da concentração da hormona ecdisona nestes processos e como é que altera a resposta quer do crescimento, quer da padronização.

Para entender estas respostas, tentámos enquadrar os melhores modelos que pudessem explicar os efeitos observados. Os modelos variavam entre um modelo linear e modelo-limite. O modelo linear é observado em várias cascatas génicas, como uma forma de aumentar ou diminuir o efeito da cascata. Já os modelos-limite são observados em várias reações que envolvem acionar ou suspender o funcionamento de cascatas génicas, funcionando como um botão bimodal.

Os nossos resultados demonstram que, no início do desenvolvimento, o crescimento dos discos imaginais não são afetados pela hormona ecdisona em fêmeas alimentadas numa dieta padrão. Já nos machos alimentados numa dieta padrão, o aumento da hormona ecdisona aumenta a taxa de crescimento dos discos. Em fêmeas que foram alimentadas apenas com sucrose, e cujas taxas de crescimento são significativamente menores, o aumento da concentração da hormona ecdisona, aumenta a taxa de crescimento dos discos até níveis semelhantes às larvas alimentadas numa dieta padrão.

Quanto à taxa de padronização, observámos que, apesar das larvas terem diferenças significativas quando alimentadas numa dieta padrão ou apenas sucrose, ambas respondem de formas semelhantes ao aumento de ecdisona. Aumentar a concentração desta hormona aciona uma resposta-limite, em vez de acionar uma resposta de aumento de taxa de padronização. O limite superior aumenta com o aumento da concentração da hormona, ou seja, aumento de ecdisona aumenta o estadio máximo que os discos imaginais conseguem atingir.

Nos últimos estadios do desenvolvimento observámos que o crescimento dos discos imaginais das asas continua sem ser afetado pela hormona ecdisona. Fornecer ecdisona a larvas alimentadas numa dieta padrão não altera significativamente a taxa de crescimento dos discos.

A padronização dos discos das asas no final do desenvolvimento tem uma resposta diferente dos estadios iniciais. As linhas controlo apresentam uma taxa de padronização exponencial, semelhante o suficiente com a resposta limite dos estadios iniciais. As larvas PGX, no entanto, apresentam uma taxa de padronização linear. Aumentar a concentração da hormona ecdisona, aumenta a taxa de padronização, mas esta mantém-se sempre linear.

Estes resultados demonstram que o tamanho da asa, que é uma característica altamente plástica, parece ser controlada por um processo robusto na sua resposta. Em fêmeas, na ausência de proteína, a hormona ecdisona pode resgatar a taxa de crescimento, mesmo que a sinalização da insulina/alvo da rapamicina (insulin/TOR) esteja inativa. A insulin/TOR é a cascata que promove crescimento em resposta a condições ambientais. A hormona ecdisona parece capaz de sobrepor a sua atividade sobre a atividade da cascata insulin/TOR para assegurar taxas de crescimento elevadas. Este processo é robusto também pelo facto de não ser alterado ao longo do desenvolvimento quando comparado com larvas alimentadas numa dieta padrão. Nos últimos estadios de desenvolvimento larvar, a hormona ecdisona continua sem produzir algum efeito em larvas alimentadas numa dieta padrão.

Por outro lado a padronização do disco imaginal da asa, que é uma característica robusta, parece ser regulada por um processo plástico. Este processo é alterado através das concentrações da hormona ecdisona. A hormona ecdisona é essencial para ativar a padronização nos discos imaginais das asas. As variações nas trajetórias de padronização que se observam em diferentes condições ambientais ou genéticas podem ser resultado duma alteração do primeiro pico de ecdisona no terceiro estadio larvar. Este pico pode ser alterado no seu tempo de aparecimento ou até mesmo na concentração de hormona libertada, ativando respostas-limite para limites diferentes, o que resulta em várias trajetórias de padronização.

No final do desenvolvimento, caso haja discos com padronização atrasada, uma rápida taxa de padronização causada por uma resposta-limite, seria uma boa estratégia para manter a robustez

desta característica. O ultimo pico de ecdisona no terceiro estadio larvar pode ser em concentrações suficientes que não só aumentam a taxa de padronização, como foi observado, mas pode eventualmente transformar uma resposta linear numa resposta-limite como a observada nos genótipos controlo.

Este estudo revelou várias interações entre o crescimento dos órgãos e a sua padronização que não eram conhecidas antes. A forma como a plasticidade e robustez de características fenotípicas é mantida através de processos de desenvolvimento plásticos ou robustos ainda tem questões por responder. Analisar estadios intermédios de desenvolvimento ou manipular a expressão de ecdisona e insulina no disco da asa levará a uma maior compreensão de como é que estas cascatas interagem com o crescimento e padronização. Levará também a um entendimento maior de como é que estas interações são diferentes entre os sexos.

Palavras-Chave: Plasticidade, Robustez, *Drosophila melanogaster*, disco imaginal da asa, crescimento, padronização, ecdisona

Index

| | |
|--|----|
| 1. Introduction..... | 1 |
| 2. Materials and Methods..... | 8 |
| 2.1. Fly Stocks and Rearing Conditions..... | 9 |
| 2.2. Larval Staging..... | 9 |
| 2.3. Dissections and Immunocytochemistry | 9 |
| 2.4. Quantification of gene pattern | 10 |
| 2.5. Statistical Analysis..... | 10 |
| 3. Results..... | 11 |
| 3.1 – Exploring the effects of ecdysone concentration in wing disc growth and patterning in early third instar stages | 12 |
| 3.2 – Exploring the relationship between ecdysone concentration, wing disc growth and wing disc patterning in late third instar stages | 21 |
| 4. Discussion..... | 30 |
| 4.1 – Exploring the effects of ecdysone concentration in wing disc growth and wing disc patterning in early third instar stages..... | 31 |
| 4.2 – Exploring the relationship between ecdysone concentration, wing disc growth and wing disc patterning in late third instar stages..... | 32 |
| 4.3 - Future Directions..... | 33 |
| 5. Conclusions..... | 34 |
| 6. Bibliography | 36 |
| 7. Appendix..... | 42 |

List of figures and tables

Figure 1.1: Achaete and Senseless differ in their relationship with disc size. A) The relationship between Achaete stage and \log_{10} Disc Area best fits a linear model. B) The relationship between Senseless stage and \log_{10} Disc Area is best fit with a threshold function: the four-parameter logistic Hill's function. In both A and B, black symbols are wild type (Samarkand) lines, grey are UAS InR parental controls, and purple are *phantom GAL4>UAS InR* larvae which are known to have increased rates of ecdysone synthesis (Mirth et al., 2009).....3

Figure 1.2: Two hypotheses for the relationship between disc size and disc pattern throughout third instar development. (A) Hypothesis 1 (Size independent): discs pattern independently of size. This would mean that discs with different growth rates (shown in black and blue in the graph) will reach the same patterning stage at different disc sizes. (B) Hypothesis 2 (Size threshold): discs have to reach a specific size for patterning to progress. Once the size threshold is reached, patterning will switch on and progress rapidly.....4

Figure 1.3: Dachshund and Senseless show differences in sensitivity to ecdysone. Larvae in which the prothoracic gland has been genetically ablated (PGX) to eliminate ecdysone production were fed on a range of ecdysone concentrations starting 55 h after third instar ecdysis. Their wing discs were then dissected 24 h later and stained for Dachshund (A) or Senseless (B). A) shows the relationship between ecdysone concentration and Dachshund stage. B) shows the relationship between ecdysone concentration and Senseless stage.....5

Figure 1.4: Alternative models explaining how developmental processes respond to ecdysone. (A) In a rate model hypothesis, increasing ecdysone will increase the growth or patterning rate across time; (B) In a threshold model hypothesis, ecdysone induces a threshold response for disc growth or patterning, which higher concentrations generating more advanced pattern.....7

Figure 3.1: Growth rates of wing discs for fed and starved control larvae. (A) Growth rates for *>grim* (black lines) and *PG>* (grey lines) females. Solid lines and circles represent fed females, dashed lines and triangles represent starved females. Starved females have lower growth rates than fed females. (B) Growth rates for *>grim* (black line) and *PG>* (grey line) males. Both controls have high growth rates.....13

Table 3.1: Comparisons in the response of \log_{10} disc area to time and genotype, for males, or time, genotype and food, for female control larvae.....14

Table 3.2: Comparisons in the response of \log_{10} disc area to time and ecdysone, for males, or time, ecdysone and food, for female PGX larvae.....14

Figure 3.2: The effect of ecdysone on growth rates of the wing discs for starved and fed PGX larvae. (A) Disc area (\log_{10}) over time for fed (solid line and circles) and starved (dashed line and triangles) female PGX larvae. (B) Disc area (\log_{10}) over time for fed PGX males. Numbers above the graphs and in the legend refer to ecdysone concentrations. (ng/mg of diet).....15

Figure 3.3: Achaete and Senseless patterning rates in wing discs of fed and starved female control larvae. (A) Achaete stage over time for *>grim* (black lines) and *PG>* (grey lines) for both fed (full lines and circles) and starved (dashed lines and triangles). (B) Senseless stage over time for *>grim* (black lines) and *PG>* (grey lines) for fed (full lines and circles) and starved (dashed lines and triangles). Both *>grim* and *PG>* starved animals have the same senseless stages.....16

Table 3.3: Comparisons in the response of Achaete stage to time and ecdysone, for male, or time, ecdysone and food, for female PGX larvae.....17

Table 3.4: Comparisons in the response of Senseless stage to time and ecdysone, for male, or time, ecdysone and food, for female PGX larvae.....17

Fig. 3.4: The effects of ecdysone concentration on Achaete patterning rates in the wing discs fed and starved PGX larvae. (A) Achaete stage over time for fed PGX females. (B) Achaete stage over time for starved PGX females. Numbers above the graphs and in the figure legends refer to ecdysone concentrations (pg/mg of diet).....18

Fig.3.5: The effect of ecdysone concentration on Senseless patterning rates in the wing discs of fed and starved PGX females. (A) Senseless stage over time for fed female PGX larvae. (B) Senseless stage over time for starved PGX females. Numbers above the graphs and in the figure legends refer to ecdysone concentrations (pg/mg of diet).....18

Fig.3.6: Achaete and Senseless patterning rates for male control larvae. (A) Achaete stage over time for *>grim* (black line) and *PG>* (grey line) males. (B) Senseless stage over time for *>grim* (black line) and *PG>* (grey line) males.....19

Fig.3.7: The effect of ecdysone concentration on Achaete and Senseless patterning rates in the wing discs of PGX males. (A) Achaete stage over time for PGX males. (B) Senseless stage over time for PGX males. Numbers above the graphs and in the figure legends refer to ecdysone concentrations (ng/mg of diet).....19

Table 3.5: Analysis of Variance Table (ANOVA) from the comparison between models, for fed and starved PGX larvae, comparing the fit of linear models versus Hill's function models for the response of Achaete stage (top) and Senseless stage (bottom) to time for a range of ecdysone concentrations.....21

Fig.3.8: Growth and patterning in the wing discs after prothoracic gland ablations (PGX) at 28 and 55 h AL3E compared to *>grim* larvae. (A) Disc area (\log_{10}) over time for PGX (green circles) and *>grim* (black circles) larvae. (B) Achaete stage over time for PGX (green circles) and *>grim* (black circles) larvae. (C) Senseless stage over time for PGX (green circles) and *>grim* (black circles) larvae. All *>grim* larvae pupariated between 55+16 and 55+24 hours AL3E.....22

Table 3.6: Comparisons in the response of \log_{10} disc area to time, sex and genotype after prothoracic gland ablation at either 28 or 55h AL3E.22

Table 3.7: Comparisons in the response of Achaete or Senseless stage to time, sex and genotype after prothoracic gland ablation at either 28 or 55h AL3E.....23

Fig.3.9: Comparing the growth and patterning of the wing discs in prothoracic gland ablations (PGX) at 55 h AL3E to control genotypes. (A) Disc area (\log_{10}) for PGX (green), *>grim* (black) and *PG>* (grey) female and male larvae. (B) Achaete stage over time in PGX (green), *>grim* (black) and *PG>* (grey) female and male larvae. (C) Senseless patterning rates for PGX (green), *>grim* (black) and *PG>* (grey) genotypes. All control larvae pupariated between 55+16 and 55+24 hours.....23

Table 3.8: Comparisons in the response of \log_{10} disc area to time, sex and genotype for prothoracic gland ablations at 55h AL3E without ecdysone supplement.....24

Table 3.9: Comparisons in the response of Achaete or Senseless stage to time, sex and genotype for prothoracic gland ablations at 55h AL3E without ecdysone supplement.....24

Fig.3.10: Growth rates in the wing discs after prothoracic gland ablation (PGX) at 55h AL3E and controls, *>grim* and *PG>*. (A) Disc area (\log_{10}) over time for PGX (green), *>grim* (black) and *PG>* (grey) female larvae. (B) Disc area (\log_{10}) over time for PGX (green), *>grim* (black) and *PG>* (grey) male larvae. Numbers above the graphs refer to ecdysone concentration (pg/mg of diet).....25

Table 3.10: Comparisons in the response of \log_{10} disc area to time, ecdysone and genotype both for females (top) and males (bottom) with 0 and 15ng/mg of ecdysone after prothoracic gland ablation at 55h AL3E.....26

F Fig.3.11: Achaete patterning rates after prothoracic gland ablation (PGX) at 55 h AL3E and in control larvae, *>grim* and *PG>*. (A) Achaete stage over time in PGX female (solid line and circles) and male (dashed line and triangles) larvae. (B) Achaete stage over time in *>grim* (black) and *PG>* (grey) female (solid lines and circles) and male (dashed lines and triangles) larvae. Numbers above the graphs refer to ecdysone concentrations (pg/mg of diet).....27

Fig.3.12: Senseless patterning rates in prothoracic gland ablations (PGX) at 55 h AL3E and controls, *>grim* and *PG>* larvae. (A) Senseless stage over time in PGX female (solid line and circles) and male (dashed line and triangles) larvae. (B) Senseless stage over time in *>grim* (black) and *PG>* (grey) female (solid lines and circles) and male (dashed lines and triangles) larvae. Numbers above the graphs refer to ecdysone concentrations (pg/mg of diet).....28

Table 3.11: Comparisons in the response of Achaete (top) or Senseless (bottom) stage to time, ecdysone and sex after prothoracic gland ablation at 55h AL3E.....29

Supplemental Figure 1: Patterning scheme from Oliveira et al., 2014. Differences in gene expression of six different genes (Achaete, Senseless, Daschund, Cut, Notch and Wingless) throughout development of *Drosophila melanogaster* from moult to L3 stage (Stage A) to white pre-pupae (Stage K). For the experiments, only Achaete and Senseless patterning progressions were used.....43

List of abbreviations and symbols

PGX – Prothoracic gland ablated

TOR – Target of rapamycin

>grim - *UAS grim*

PG> - *phmGAL4*

EGF – Epidermal growth factor

MS – Methionine synthase

MTHFR – Methylene tetrahydrofolate reductase

DNA - Desoxyribonucleic acid

DNMT – DNA methylation reaction

Dpp – Decapentaplegic

AL3E – After L3 ecdysis

SYA – Sugar-yeast-agar

g – gram

°C – Celsius degree

PBS – Phosphate-buffered saline

PBT – Phosphate-buffered saline with Triton

ANOVA – Analysis of variance

ng – nanogram

mg – milligram

dILP – *Drosophila* insulin like peptides

mRNA – Messenger ribonucleic acid

1. Introduction

It's better to know how to learn than to know – Dr. Seuss

All environments are dynamic, either temporally or spatially. Because of this, animals and plants have mechanisms that allow them to adjust to their environmental conditions in order to survive. Depending on the trait in question, organisms can change their phenotypes to better fit their environment, known as phenotypic plasticity (Moczek et al., 2011), or they can have inbuilt mechanisms that allow them to maintain phenotypes constant over a range of environmental conditions, called robustness. Whether a trait changes or remains constant in response to environmental variation depends on the nature of the trait in question. Further, a single organ can exhibit both plastic and robust traits. A multitude of studies have uncovered the genetic mechanisms that allow traits to be plastic in response to environmental variation. Similarly, both theoretical and genetic studies have demonstrated mechanisms underlying trait robustness. However, few studies have explored how organs coordinate their development to allow plasticity in some traits, while maintaining the robustness of others.

In an environment in constant change, plasticity is vital for survival. Phenotypic plasticity allows an organism, with its own singular and unique genotype, to present a variety of phenotypes, each of which depends on the environmental context during development. Phenotypic plasticity has been documented in virtually every type of organism, from plants such as *Hydrangea macrophylla* that changes petal colours depending on the amount and kind of heavy metals in the soil (Oyama et al., 2015), to mammals, such as foxes that change fur colour from orange to white depending on the season (Hart, J. 1956). Being able to express these plastic traits is essential to cope with a temporally or spatially changing environment.

Many of the most impressive examples of phenotypic plasticity are found in insects. Social insects in particular present striking examples, remodelling their development in response to key environmental variables. Ant species such like *Pheidole rhea* develop into one of several castes, either workers, soldiers, or queens, depending on the nutrition they are given in their growth phase, the larval stage (Huang et al., 2013). In the African butterfly *Bicyclus anynana*, seasonal cues during development alter the colour patterns of the wings to generate large eyespots in the wet season and small eyespots in the dry (Brakefield et al., 2007). Although these insects present dramatic examples of plasticity, understanding the genetic mechanisms regulating this plasticity is limited by the narrow range of genetic tools available in these species.

The molecular mechanisms underlying plasticity have been best described in the fruit fly *Drosophila melanogaster*, thanks to its impressive genetic toolkit. Although *D. melanogaster* does not present the dramatic alterations in body size and shape found in ants, plasticity in body/organ size induced by changing thermal and nutritional environments has received significant attention over the past 15 years. In *D. melanogaster*, body and organ size varies with nutritional availability (Beadle et al., 1938, Robertson, 1963). Nutrition affects body size in two ways, by regulating growth rate or regulating the duration of growth (Nijhout 2003; Mirth & Riddiford, 2007; Shingleton et al 2008).

The insulin/target of rapamycin (TOR) pathway, conserved in all animals, regulates growth rates of the body and its organs in response to nutrition (Shingleton, 2010). In addition, insulin/TOR regulates the duration of growth by controlling the timing of an important developmental transition known as critical weight (Shingleton et al 2005, Mirth et al 2005, Koyama et al 2014). Animals starved before critical weight delay the onset of metamorphosis. After critical weight, starvation induces mild acceleration in pupariation.

Insulin/TOR regulates this transition by acting on the prothoracic gland, the gland responsible for producing the steroid moulting hormone ecdysone. Ecdysone peaks three times during the third instar before a fourth peak induces pupariation (Warren et al, 2006). Reducing insulin/TOR signalling

in the prothoracic gland represses the first of these peaks, thereby extending the larval phase (Mirth et al 2005, Koyama et al 2014).

In addition to its effects on developmental timing, the ecdysone peak that induces critical weight also alters the effect of nutrition on organ growth. Starving *D. melanogaster* larvae of yeast before critical weight represses growth of both the wing imaginal disc, the pouch of cells that will give rise to the adult wing and notum, and the developing ovary more than starvation after critical weight (Shingleton et al., 2008, Mendes & Mirth, 2016). This is not simply a peculiarity of *D. melanogaster* wing disc growth. In *Manduca sexta*, growth in the wing imaginal discs is more sensitive to nutrition before critical weight than after (Tobler & Nijhout 2010).

In the ovary of *D. melanogaster*, differences in the effect of nutrition before and after critical weight result from a change in how ovary growth is regulated (Mendes & Mirth, 2016). Whereas insulin/TOR regulates most of the growth of the ovary before critical weight, both insulin/TOR and ecdysone contribute to ovary growth after (Mendes & Mirth 2016). A similar mechanism is thought to act in the wing disc, as ecdysone plays an important role in regulating the growth of wing discs after critical weight (Ghosh et al., 2013, Herboso et al., 2015).

Whereas the ability to alter phenotypes like organ or body growth in response to environmental conditions is clearly beneficial, other phenotypes need to remain the same across different environments. This is called phenotypic stability or robustness (Nijhout et al. 2017). Examples of this include many types of enzymatic reactions. Substrates can be converted to their final compounds via several pathways, each pathway with its own unique properties (Felix and Wagner, 2006). Since this conversion starts from the same substrate and ends with the same product, these alternative pathways convey robustness to this process. This becomes important when one branch of an enzymatic cascade fails to function, due to a mutation or lack of cofactors. Alternative branches of this pathway that can execute the same reaction ensure the final product is produced, vital if this product is necessary for proper cell function. Thus, phenotypic robustness exists not due to an unchangeable state, but because the properties of systems change dynamically to ensure the phenotype remains constant.

Other types of processes generate phenotypic robustness. Dominance in heterozygotes is also an example of robustness, since having either one or two copies of a gene results in the same

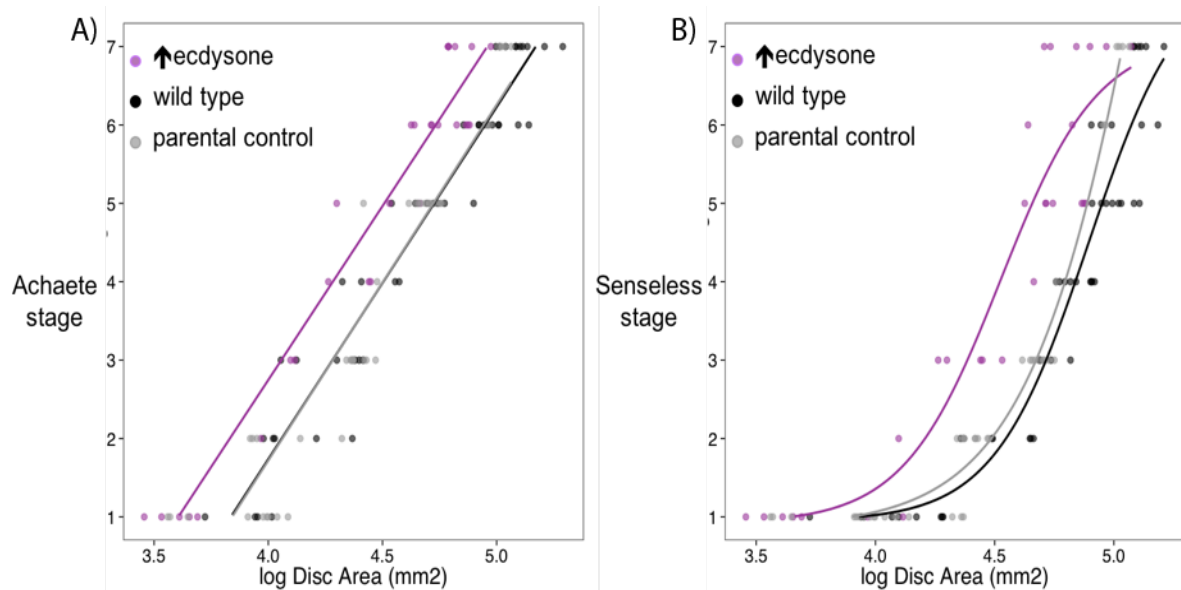


Figure 1.1: Achaete and Senseless differ in their relationship with disc size. A) The relationship between Achaete stage and \log_{10} Disc Area best fits a linear model. B) The relationship between Senseless stage and \log_{10} Disc Area is best fit with a threshold function: the four-parameter logistic Hill's function. In both A and B, black symbols are wild type (Samarkand) lines, grey are UAS InR parental controls, and purple are *phantom GAL4>UAS InR* larvae which are known to have increased rates of ecdysone synthesis (Mirth et al., 2009).

phenotype (Kacser and Burns, 1981), for example brown eyes result from a single copy of the allele that confers brown colour to eyes. Another example, in *Caenorhabditis elegans* the vulva's final cell fate pattern is highly robust. LIN-3/epidermal growth factor (EGF) is necessary for specifying the cells of the vulva (Hill & Sternberg, 1992). A four-fold reduction in genetic dosage of LIN-3 still results in proper vulval induction (Barkoulas, et al., 2013). Finally, in *D. melanogaster* the genital disc changes little in size relative to the rest of the body across thermal or nutritional gradients (Shingleton et al., 2009). This occurs because the pathway that makes organs sensitive to nutritional variation, the insulin/TOR signalling pathway, is altered specifically in this organ such that insulin/TOR signalling remains high in this tissue even when the animal is starved (Tang et al., 2011).

Even for traits that are robust, the capacity for phenotypic robustness is not indefinite. Although robust traits tend to vary little within a range of genetic backgrounds or environmental conditions, outside this range phenotypic variability can increase rapidly (Nijhout et al., 2014). This is the case of the methionine synthase (MS) and methylenetetrahydrofolate reductase (MTHFR) enzymes, both involved in the DNA methylation reaction (DNMT). Genetic variation can reduce the activity of these two enzymes up to 30% without significantly altering the rate of the DNMT reaction. However, when faced with an environmental change in the form of a deficiency for vitamin B12, a cofactor for MS, genotypes with reduced MS or MTHFR activity are unable to maintain constant rates of DNMT reactions (Nijhout et al., 2017). This shows that the range of conditions that a process remains robust depends on complex interactions between genetic variation and environmental conditions.

Although these studies explore the mechanisms and limitations of trait robustness, they do not address how single organs coordinate developmental processes that lead to trait plasticity, like

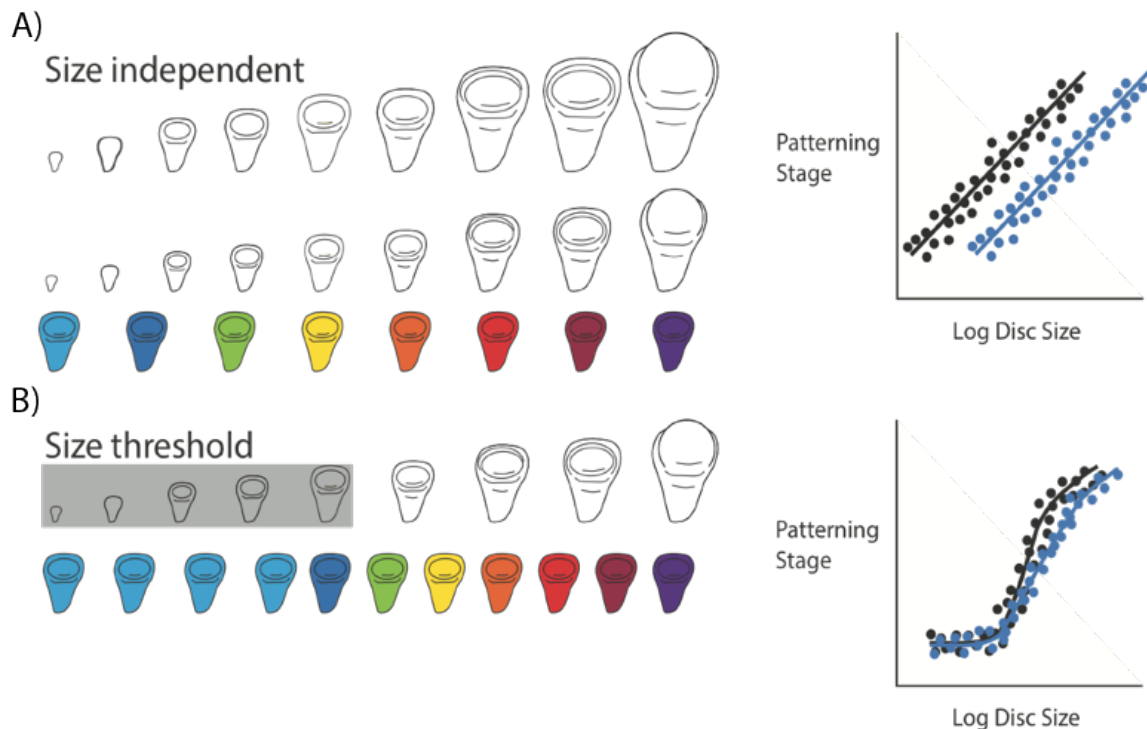


Figure 1.2: Two hypotheses for the relationship between disc size and disc pattern throughout third instar development. (A) Hypothesis 1 (Size independent): discs pattern independently of size. This would mean that discs with different growth rates (shown in black and blue in the graph) will reach the same patterning stage at different disc sizes. (B) Hypothesis 2 (Size threshold): discs have to reach a specific size for patterning to progress. Once the size threshold is reached, patterning will switch on and progress rapidly.

growth, with those that lead to trait robustness, like patterning. The wings of *D. melanogaster* may vary in size, depending on the developmental environment of the larva, but the structures of the wing, such as the vein number and position and the position of sensory bristles, are invariant. These structures are not established by a single robustness gene, but rather by a series of patterning gene products, such as Achaete, Senseless, and Decapentaplegic (Dpp) (Restrepo et al., 2014), that interact with each other and also with hormones, such as ecdysone (Nijhout et al., 2007), to specify cell fates. In *D. melanogaster*, the robustness of patterning in wing imaginal discs is determined by several factors, one of them a gradient of Dpp, that acts as a morphogen to pattern the organ (Teleman & Cohen 2000). Without this Dpp gradient, the wing imaginal disc is unable to activate the proper genes to progress in its patterning, and therefore will not develop into a functional wing. Further, hormones, most notably ecdysone, ensure that patterning events occur at the appropriate time in the wing (Mirth et al., 2009, Schubiger & Truman 2000, Schubiger et al., 2005). Thus, the regulation of pattern at several levels ensures that at the end of development the wing is correctly patterning, bearing all the correct structures and ensuring the robustness of this trait.

Although the final pattern of organs like the wing remains constant across environmental conditions and across wild type genetic backgrounds, the rate of patterning has been shown to vary with environmental conditions, physiological conditions, and genetic background. Starving larvae of yeast before they reach critical weight induces substantial delays in the patterning of both the wing and the ovary (Mirth et al., 2009, Mendes and Mirth, 2016). The peak of ecdysone at critical weight mediates this transition. Activating ecdysone signalling in the wing discs or ovaries of yeast-starved larvae allows organ patterning to progress (Mirth et al., 2009, Mendes and Mirth, 2016). These findings highlight that even though the final pattern is robust, the trajectory to reach that final state can be highly plastic and is affected by ecdysone signalling.

Given that wing size and wing pattern are both regulated by ecdysone during larval development, how are the developmental processes that govern each coordinated to ensure that wings

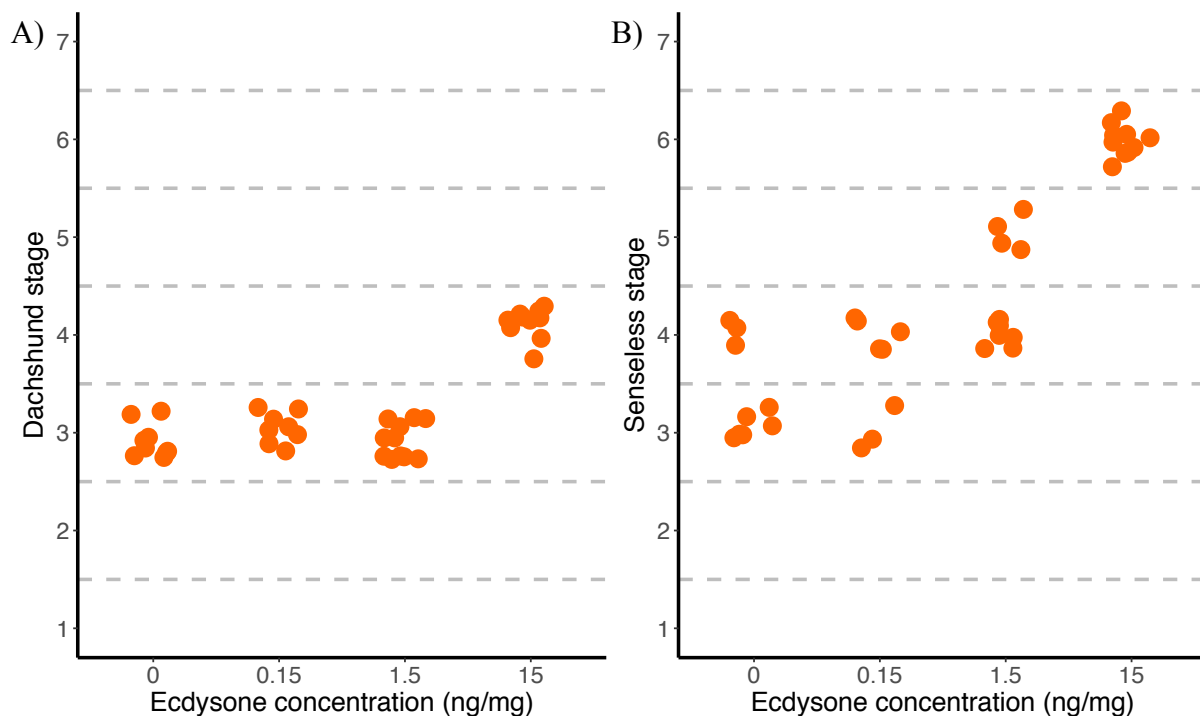


Figure 1.3: Dachshund and Senseless show differences in sensitivity to ecdysone. Larvae in which the prothoracic gland has been genetically ablated (PGX) to eliminate ecdysone production were fed on a range of ecdysone concentrations starting 55 h after third instar ecdysis. Their wing discs were then dissected 24 h later and stained for Dachshund (A) or Senseless (B). A) shows the relationship between ecdysone concentration and Dachshund stage. B) shows the relationship between ecdysone concentration and Senseless stage.

are correctly patterned independent of their size? One approach to understanding how plastic and robust developmental responses are coordinated is to examine the extent to which patterning is coordinated with other developmental processes, such as organ growth or developmental time of the whole body. In the buckeyed butterfly, *Precis coenia*, wing disc patterning, as measured by the extent of lacuna development and of tracheation, occurs independently of wing disc growth (Miner et al., 2000). These studies would lead us to conclude that patterning and growth in the wing disc are independent processes.

More recently, Oliveira and colleagues (2014) tested to what extent patterning of the wing disc was coordinated with developmental events that regulate developmental timing across the whole body in *D. melanogaster*. To do this, they developed a system for quantifying the patterning stage of the wing disc using the changes in expression domains of six patterning gene products, including Achaete and Senseless (Oliveira et al., 2014) (Figure 1.1). They calibrated their scheme to whole body developmental events: moulting to third instar (L3 stage), wandering, when the larvae emerge from the food to find a pupariation site, and pupariation. They then generated environmental or hormonal variation, by changing temperature or examining genotypes that varied in their rates of ecdysone synthesis. Comparing the rates of patterning across these conditions, they found that patterning stage was invariant at two developmental events: at the moult to the third instar and at pupation (Oliveira et al., 2014). Between these events, the rates of patterning varied significantly. This study illustrates that although patterning does not depend strictly on the mechanisms that regulate developmental time throughout the entire third instar, it is coordinated with whole body development at key developmental milestones.

From this study, two questions arose: a) what generates variation in patterning and b) given the variation in patterning rates, what ensures that patterning is invariant at pupariation? As a first attempt to understand what gives rise to variation in patterning rate, we explored the relationship between disc size and disc patterning. Because we know that disc size and pattern both rely on ecdysone signalling, variation in patterning rate across conditions might be generated by variation in disc growth across the third instar. This led to two hypotheses: 1) that disc pattern was independent of disc size, as has been described for *P. coenia*, (Fig 1.2A) or 2) that discs need to attain a minimal threshold size for patterning to progress (Fig 1.2B).

Preliminary data collected by Dr. Marisa Oliveira suggests that the relationship between disc size and disc pattern differs between patterning gene products (Fig1.1). When comparing the disc size/disc pattern relationships in the wing disc using two patterning gene products, Achaete and Senseless, Dr. Oliveira found that Achaete stage increases linearly with increasing size (Fig. 1.1A). Increasing the rate of ecdysone synthesis shifted the disc size/disc patterning relationship to the right but the relationship remained linear (Fig.1.1A). This suggests that Achaete patterning progresses independently of disc size. Senseless, on the other hand, shows a threshold response, where patterning only occurs once the disc has reached a certain size (Fig. 1.1B). Increasing the rate of ecdysone synthesis shifts the disc size threshold to earlier in development, meaning that wing imaginal discs switch on patterning at smaller sizes (Fig.1.1B). These data support a size threshold model for the disc size/disc pattern relationship. These data suggest two things: 1) that patterning gene products differ in their relationship with wing disc size and 2) that ecdysone regulates patterning progression for both Achaete and Senseless. This difference in how Achaete and Senseless respond to disc size and to ecdysone potentially highlights a mechanism for generating variation in patterning rate.

The second question arising from the Oliveira et al (2014) study is once variation in patterning is generated, how do discs alter their rates of patterning to ensure that they all reach the same final stage at pupariation? Dr. Oliveira's preliminary data suggest that ecdysone may play a role

in ensuring that all discs reach the correct patterning stage at pupariation. She genetically ablated the prothoracic gland (PGX), to eliminate ecdysone production and fed these larvae a range of concentrations of ecdysone starting at 55 h after third instar ecdysis (AL3E) (Figure 1.3). She then dissected out the discs and performed immunocytochemistry for two patterning gene products, Dachshund and Senseless. She found that Senseless required less ecdysone to change between stages than Dachshund (Figure 1.3). We thus hypothesized that both the variation in patterning rate generated at earlier stages of development and the coordination of pattern at the end of development both depend on how patterning gene products respond to ecdysone.

To test these hypotheses, we explored how wing disc growth and wing disc patterning responded to concentrations of ecdysone at both early stages and later stages of the third instar. We compared how disc growth and disc patterning responded to ecdysone using one of two models. Ecdysone could either regulate the rates of growth and patterning, with changes in ecdysone changing the slope of this relationship (Fig. 1.4A) or growth and patterning could respond to ecdysone in a threshold manner (Fig. 1.4B). This would mean that the lowest ecdysone concentrations would not induce patterning, but higher amounts of ecdysone would trigger a switch-like response (Fig. 1.3B). Changes in how disc growth rates and patterning gene products respond to ecdysone across developmental stage could explain how variation in patterning rate early and coordination of patterning late in the third instar occur. Furthermore, differences in how disc growth and disc patterning respond to ecdysone will highlight how the same developmental hormone can generate both plastic and robust traits within the same organ. Taken together, these studies will shed insight into how plasticity and robustness are coordinated to give rise to a fully functional organs or organisms across a range of environmental conditions.

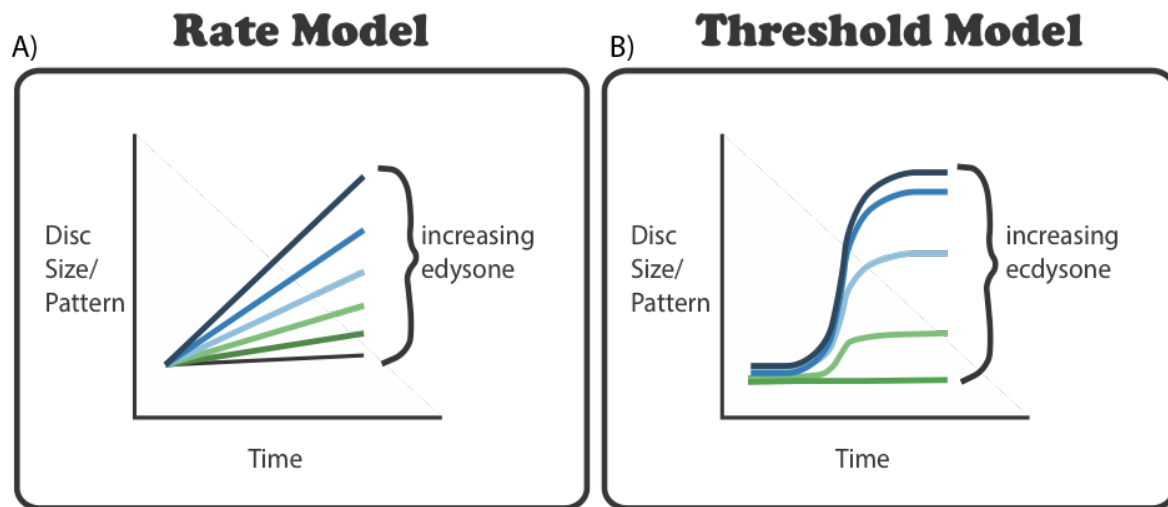


Figure 1.4: Alternative models explaining how developmental processes respond to ecdysone. (A) In a rate model hypothesis, increasing ecdysone will increase the growth or patterning rate across time; (B) In a threshold model hypothesis, ecdysone induces a threshold response for disc growth or patterning, which higher concentrations generating more advanced pattern.

2. Materials and Methods

You have brains in your head. You have feet in your shoes.
You can steer yourself any direction you choose. – Dr. Seuss

Fly Stocks and Rearing Conditions

We used a transgenic combination that allowed us to ablate the prothoracic gland to eliminate native ecdysone synthesis specifically in the third larval instar. We crossed the progeny from *tub-GAL80^{ts}*, *phm GAL4* with *UAS Grim* (*phm>Grim*, *PGX*) (Herboso et al., 2015). *GAL80^{ts}* is a repressor of *GAL4* active at temperatures lower than 22°C (Suster et al., 2004). Rearing larvae at 17°C maintain *GAL80^{ts}* activity, ensuring that the *phmGAL4*, expressed specifically in the prothoracic gland, cannot drive the expression of *UAS grim*, a cell death gene. Under these conditions, the prothoracic gland does not undergo programmed cell death, and the larvae can moult, pupariate, and complete metamorphosis (Herboso et al., 2015). Changing the larval rearing temperature to 29°C disables *GAL80^{ts}* activity, thus ablating the prothoracic gland. The progeny of an isogenic wild-type strain, *w¹¹¹⁸*, crossed with one of two parental lines, either *phm-GAL4* (*PG>*) or *UAS Grim* (*>grim*), were used as controls for genetic background effects. The parental controls were reared under the same thermal conditions as *PGX* larvae.

Crosses, egg collections, and larval rearing were done on Sugar-Yeast-Agar (SYA) medium with 20% extra water. This medium consists of 50g of autolysed Brewer's yeast powder (MP Biomedicals), 100 g of sugar, 10 g of agar and 1200 ml of water. In addition, we added 3 ml of propionic acid and 3 grams of nipagen to prevent bacterial and fungal growth.

Egg collections were performed on SYA medium for 4 hours at 25°C or overnight at 17°C and larvae were reared at controlled densities of 200 eggs per food plate (60 x 15 mm Petri dish filled with SYA medium) at 17°C, as described previously (Herboso et al., 2015).

Larval Staging

For the *PGX*, *PG>*, and *>Grim* genotypes, larvae were raised from egg to second instar at 17°C. Larvae were staged into 2-hour cohorts at ecdysis to the third larval instar by flooding the food plate with 20% sucrose and transferring all the second instar larvae to a new food plate. After two hours, the food plate was flooded once again with 20% sucrose and the newly moulted third instar larvae were collected and transferred to food plates at 29°C at the desired time interval.

For early shift experiments, newly ecdysed larvae were separated into males and females, and then transferred at 0 h AL3E to 29°C and fed either a SYA medium or a 20% sucrose in 1% agar medium without additional yeast. Larval wing discs were sampled at the following times: 0, 5, 10, 15, 20, 25, 30h AL3E. The active form of ecdysone, 20-hydroxyecdysone (Sapphire Bioscience, catalogue #16145), was added to the different foods in the following concentrations (in ng/mg of SYA or sucrose medium): 0, 6.25, 12.5, 25, 50, 100 ng/mg of food.

For late shift experiments, newly ecdysed L3 larvae were separated into males and females and returned to 17°C for an additional 28 or 55 h, after which they were transferred to 29°C for the following times (in h AL3E): 28+0, 28+24, 55+0, 55+8, 55+16, 55+24h AL3E. Ecdysone was added to the food in the following concentrations: 0, 0.15, 1.5, 15ng/mg of food.

Dissections and Immunocytochemistry

For each time point sampled, at least 10 larvae were dissected in ice cold PBS and fixed in 4% formaldehyde in PBS overnight at 4°C. The number of discs varied depending on the treatment/genotype, ranging from 5 to 26 discs. After fixation, the tissue was washed four times (15 minutes per wash) with 0.3% Triton X-100 in PBS (PBT), then blocked for 30 minutes at room temperature in 2% heat-inactivated normal donkey serum in PBT. After blocking, the tissue was incubated in a primary antibody solution diluted with 2% heat-inactivated normal donkey serum in PBT overnight at 4°C. The washing and blocking procedure was repeated after primary antibody incubation and then the tissue was incubated in a secondary antibody overnight at 4°C. The tissues were washed with PBT and rinsed with PBS and then the wing imaginal discs were mounted on poly-L-lysine-coated coverslips using Fluoromount-G (SouthernBiotech). Tissues were imaged using a Nikon confocal microscope and processed using ImageJ (version 2.0) and Adobe Photoshop CC 2017.

The primary antibodies used to stain wing-imaginal discs were: anti-Senseless (Nolo et al., 2000, 1:1000) and anti-Achaete (supernatant, 1:10). The secondary antibodies used were: anti-guinea pig (546nm) and anti-mouse (488nm). To compare signal across tissues, we stained for both antigens simultaneously.

Quantification of gene pattern

The expression stage of each of the gene products was quantified using the staging scheme developed by Oliveira et al., 2014, associating each of the wing imaginal discs to an Achaete or Senseless stage varying from 1 to 7 according to its expression pattern (Supplemental Figure 1).

For wing disc size, the wing disc area was measured by defining the disc perimeter, through use of the software ImageJ (version2.0).

Statistical Analysis

For all experiments, the data were first fit with linear models using either \log_{10} -transformed disc area or gene stage as the dependent variable, time as the independent variable and factors such as sex, genotype, food, and ecdysone concentration as fixed effect factors. To test between the rate and the threshold models for growth and patterning, we fit the data with both linear models and four parameter logistic (Hill's function) models. We used ANOVA to test whether the logistic models significantly improved the fit to the data. Data analysis and statistics were conducted using RStudio (version 1.0.143).

3. Results

Sometimes the questions are complicated
and the answers are simple – Dr. Seuss

The primary aim of this study was to determine how ecdysone controls both plasticity of growth while maintaining robustness of pattern. To address this question, we examined the effects of ecdysone on the growth and patterning of the wing discs both at the onset of third instar development, where growth and patterning rates show sensitivity to nutrition, and at the end of the third instar, when growth ceases and patterning stage becomes invariant across environmental conditions. By comparing how wing discs respond to ecdysone at these two stages of development we can gain insight into how variation in patterning and growth is generated, and into how patterning is coordinated at the end of larval development.

3.1 – Exploring the effects of ecdysone concentration in wing disc growth and patterning in early third instar stages

To assess the effect of ecdysone on growth and patterning at the onset of the third instar, we generated variation in growth rates of the disc by rearing larvae on one of two different diets, standard food (SYA medium) and a starvation medium consisting of 20% sucrose (w/v) and 1% agar, which starves larvae of yeast but does not deprive them of calories. We analysed wing disc growth and wing disc patterning separately for male and female larvae, to account for known differences in disc growth between sexes (Testa et al., 2013). Control genotypes, *>grim* and *PG>*, were staged at the moult to third instar then separated into males and females. Females were reared on either standard food or starvation medium, males were reared on standard food alone. PGX larvae were also staged to the third instar then separated into males and females. We performed more detailed analysis on female larvae, using the males as a comparison. Females were transferred to either standard food or starvation medium supplemented with one of six different ecdysone concentrations (0, 6.25, 12.5, 25, 50, 100 ng/mg of diet). Males were only transferred to standard food, and this food was supplemented with one of the six concentrations of ecdysone outlined above. For all genotypes, we collected wing discs at defined intervals between 0 and 30 hours after the moult, processing these discs for immunocytochemistry against Achaete and Senseless to assess the progression of pattern.

The effects of ecdysone concentrations on wing disc growth early in the third instar

Wing imaginal discs are known to grow exponentially in the early third instar. To be able to compare disc growth rates across conditions and genotypes, we \log_{10} transformed disc area, thereby linearizing the data, and examined the relationship with \log_{10} disc area over time. We then used ANOVA to compare disc growth between genotypes and conditions. Significant interactions between either diet type and time or ecdysone concentration and time indicate differences in disc growth rates between these conditions.

For the control genotypes, *>grim* and *PG>*, under standard food conditions the discs grow linearly with time for both the female and male larvae (Fig. 3.1). However, in starved female larvae wing discs do not grow, differing significantly in growth rate from wing discs from fed females (Fig. 3.1, Table 3.1). These results are similar to previously published data showing that starving larvae early in the third instar, before they have reached critical weight, impedes most disc growth. (Shingleton et al., 2008). For females there is a slight but significant effect of genotype on growth rates, and the interaction between time, genotype, and diet type is significant (Fig. 3.1, Table 3.1). For males, there is no difference in growth rate between genotypes on the standard food (Fig. 3.1, Table 3.1).

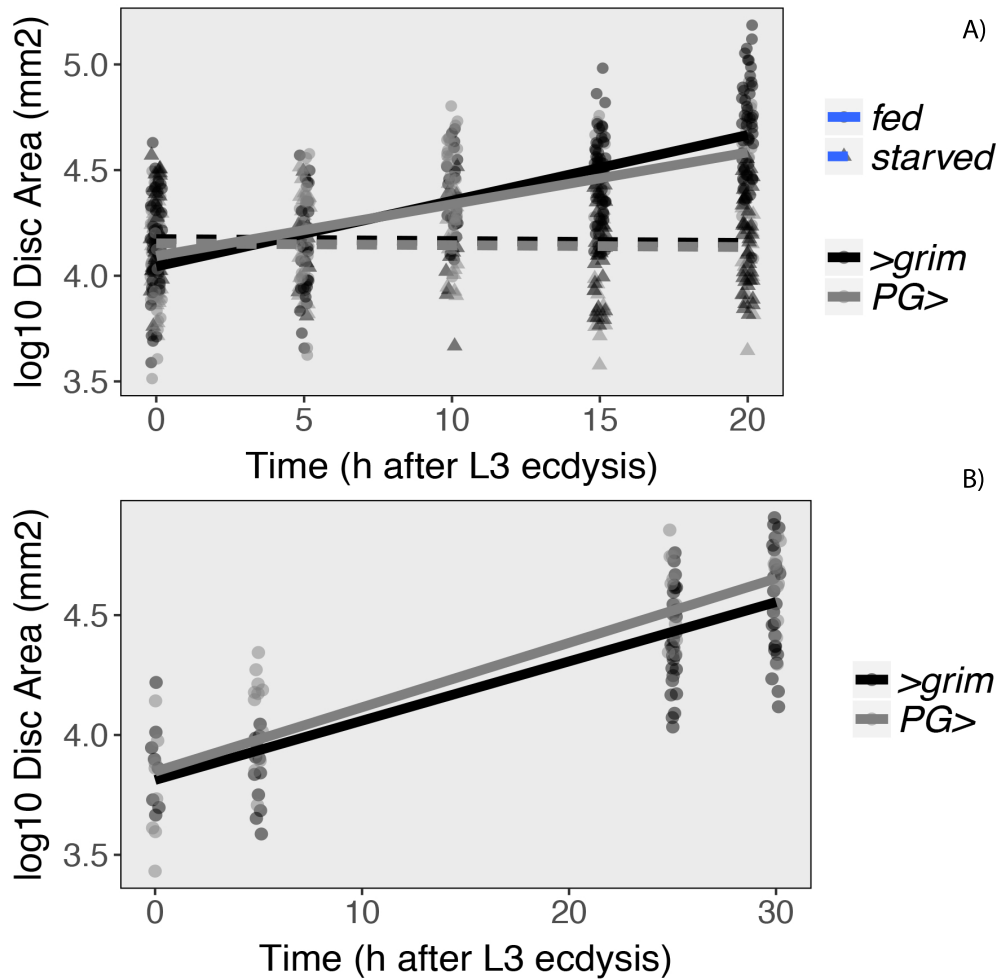


Figure 3.1: Growth rates of wing discs for fed and starved control larvae. (A) Growth rates for >grim (black lines) and PG> (grey lines) females. Solid lines and circles represent fed females, dashed lines and triangles represent starved females. Starved females have lower growth rates than fed females. (B) Growth rates for >grim (black line) and PG> (grey line) males. Both controls have high growth rates.

We next explored how ecdysone concentrations affect the growth of the wing discs in the PGX larvae. In fed PGX females, discs increase in size with time (Fig. 3.2, Table 3.2). However, growth rates are unaffected by ecdysone concentration in fed female discs (Fig. 3.2, Table 3.2). In contrast, PGX females raised on starvation medium without ecdysone supplement showed significantly slower growth rates than fed PGX females, resulting in a significant interaction between time and diet treatment (Fig. 3.2, Table 3.2). In the starved larvae, disc growth rate increased with the rising ecdysone concentrations (Fig. 3.2, Table 3.2), approaching growth rates similar to fed discs at the highest ecdysone concentrations.

In contrast, wing discs from fed PGX males increased their rates of growth with increasing ecdysone concentrations, resulting in a significant interaction between ecdysone concentration and time (Fig. 3.2, Table 3.2). Thus, at least for in the standard food conditions males and females differ in the way the growth of their wing discs responds to ecdysone.

Table 3.1: Comparisons in the response of \log_{10} disc area to time and genotype, for males, or time, genotype and food, for female control larvae.

| Response: \log_{10} disc area | Sums of Squares | Df | F-value |
|------------------------------------|--------------------|----------|------------------|
| Females | | | |
| Time | 8.20 | 1 | 493.10*** |
| Diet | 5.05 | 1 | 303.69*** |
| Genotype | 0.02 | 1 | 1.65 |
| Time*Diet | 6.60 | 1 | 396.76*** |
| Time*Genotype | 0.11 | 1 | 6.80** |
| Diet*Genotype | 0.00 | 1 | 0.10 |
| Time*Diet*Genotype | 0.09 | 1 | 5.13* |
| Residuals | 9.68 | 582 | |
| Males | | | |
| Time | 0.14 | 1 | 10.93** |
| Genotype | 0.02 | 1 | 1.36 |
| Time*Genotype | 0.00 | 1 | 0.01 |
| Residuals | 0.45 | 36 | |

Females: $F_{7,582}=179.1$, p value<0.001, Adjusted $R^2=0.68$

Males: $F_{3,36}=4.10$, p value=0.013, Adjusted $R^2=0.19$

Significance code: * ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.001 , significant parameters are highlighted in bold.

Table 3.2: Comparisons in the response of \log_{10} disc area to time and ecdysone, for males, or time, ecdysone and food, for female PGX larvae.

| Response: \log_{10} disc area | Sums of Squares | Df | F-value |
|------------------------------------|--------------------|----------|-------------------|
| Females | | | |
| Time | 22.01 | 1 | 1197.25*** |
| Ecdysone | 0.23 | 1 | 12.36*** |
| Food | 0.63 | 1 | 34.35*** |
| Time*Ecdysone | 0.06 | 1 | 3.26 |
| Time*Food | 1.47 | 1 | 80.22*** |
| Ecdysone*Food | 0.13 | 1 | 7.29** |
| Time* Ecdysone*Food | 0.24 | 1 | 12.97*** |
| Residuals | 27.58 | 1500 | |
| Males | | | |
| Time | 8.82 | 1 | 545.27*** |
| Ecdysone | 1.03 | 1 | 63.93*** |
| Time*Ecdysone | 0.09 | 1 | 5.84* |
| Residuals | 3.85 | 238 | |

Females: $F_{7,1500}=196.5$, p value<0.001, Adjusted $R^2=0.48$

Males: $F_{3,238}=209$, p value<0.001, Adjusted $R^2=0.72$

Significance code: * ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.001 , significant parameters are highlighted in bold.

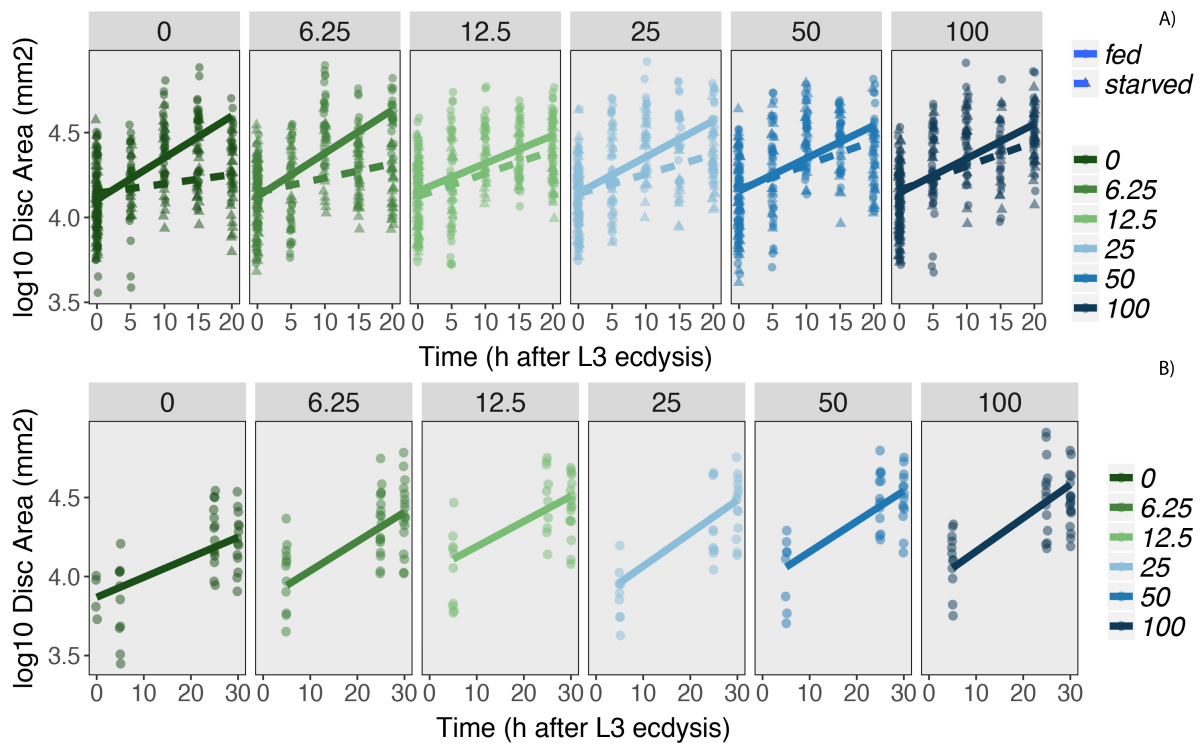


Figure 3.2: The effect of ecdysone on growth rates of the wing discs for starved and fed PGX larvae. (A) Disc area (\log_{10}) over time for fed (solid line and circles) and starved (dashed line and triangles) female PGX larvae. (B) Disc area (\log_{10}) over time for fed PGX males. Numbers above the graphs and in the legend refer to ecdysone concentrations. (ng/mg of diet)

The effects of ecdysone concentrations on wing disc patterning early in the third instar

We next examined the progression of patterning for Achaete and Senseless in control larvae. In both control genotypes, Achaete progressed steadily throughout the third instar, starting from stage 1 at 0 h after the third instar moult and reaching stages 5 and 6 by 30 h after the moult (Fig. 3.3A). Senseless expression progressed more slowly, remaining at stage 1 until 15 h after the moult. After 15 h, Senseless began to increase in stage, reaching stages 4 and 5 by 30 h (Fig. 3.3B). In both cases, patterning of the wing discs in fed males showed similar dynamics (Fig. 3.6). For the starved female larvae, patterning fails to progress within the first 20 h after the moult, with all discs remaining at stages 1 or 2 for Achaete and stage 1 for Senseless. These results corroborate previous studies by Mirth et al (2009), demonstrating that starving larvae before critical weight inhibits patterning in the wing disc.

Then, we determined how ecdysone concentration regulated the progression of pattern in PGX larvae. In female larvae, the rate of patterning significantly affected by ecdysone concentration for both patterning gene products (Figures 3.4 & 3.5, Time * Ecdysone interactions in Tables 3.3 & 3.4). Although the interaction term between diet and time was not significant for either gene product, the interaction between diet and ecdysone and the three way interaction between time, diet and ecdysone concentration was significant for both Achaete and Senseless. This indicates that the effect of ecdysone on Achaete and Senseless patterning rates depends on whether the larvae were fed or starved. Similarly, in male larva ecdysone significantly affected the patterning rates for both Achaete and Senseless (Fig. 3.7 and Tables 3.3 & 3.4).

For Achaete patterning, discs from fed PGX female larvae pattern slowly in the absence of ecdysone, reaching stages 3 and 4 by 30 h after the moult (Fig. 3.4). In starved PGX females, Achaete does not surpass stage 2 in the absence of ecdysone. Adding ecdysone to the medium increases the upper boundary, with discs reaching stages 5 and 6 by 30 h after the moult in fed females. Changing the concentration of ecdysone appears to change the speed at which these discs reach later stages of Achaete progression. At the highest ecdysone concentrations (100 ng/mg), some discs reach stage 5 as early as 10 hours after the moult. Male discs appear to respond in a similar manner, however we have insufficient data to resolve the dynamics of Achaete stage over time. Taken together, although Achaete patterning progresses to a certain extent on its own in PGX larvae, ecdysone appears to regulate the rate at which patterning occurs.

In PGX females that have not been supplemented with ecdysone, Senseless pattern does not progress beyond stage 2 by 30 h after the moult in fed larvae or beyond stage 1 by 20 h after the moult in starved larvae (Fig. 3.4B). Similarly to Achaete, adding ecdysone to the medium increases the patterning stage in both the fed and starved conditions, with the highest ecdysone concentrations resulting in discs that reach stages 4 and 5 by 30 h after the moult in fed larvae. Senseless male discs respond to increasing ecdysone concentrations in a similar manner. These data indicate that patterning rates for Senseless depend on ecdysone concentration.

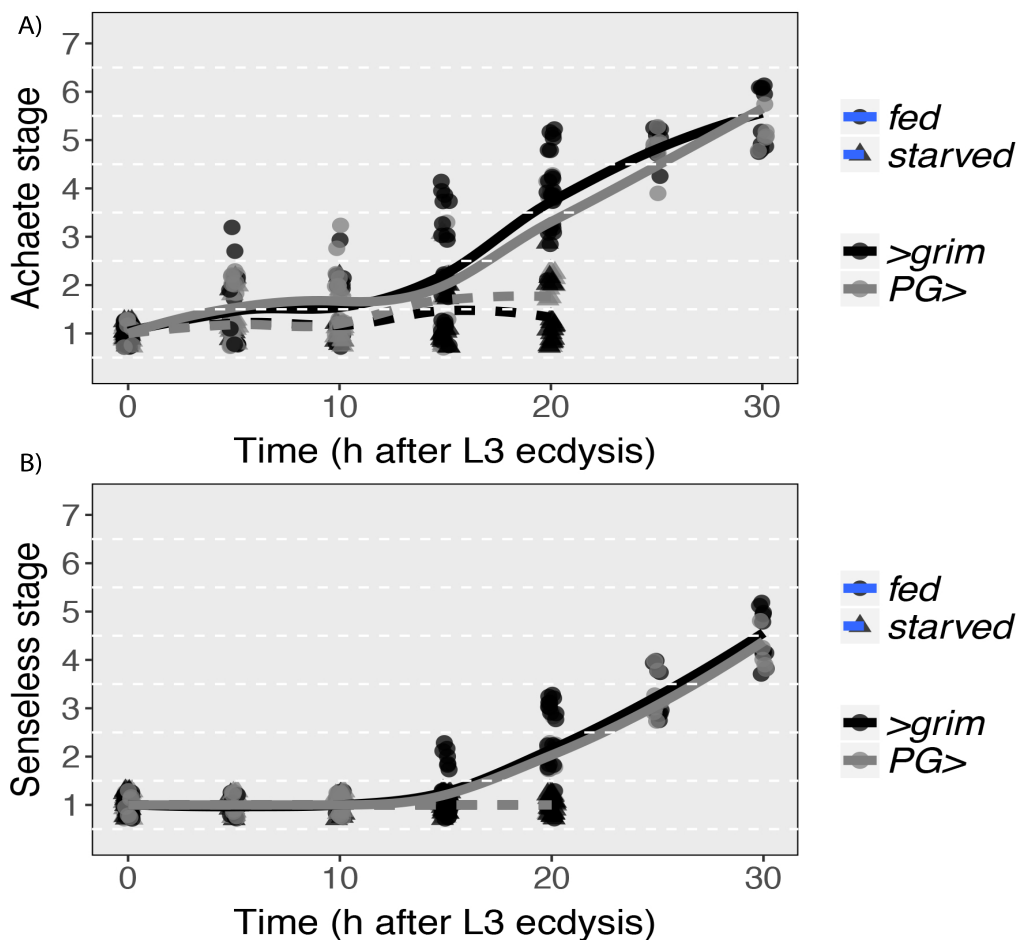


Figure 3.3: Achaete and Senseless patterning rates in wing discs of fed and starved female control larvae. (A) Achaete stage over time for *>grim* (black lines) and *PG>* (grey lines) for both fed (full lines and circles) and starved (dashed lines and triangles). (B) Senseless stage over time for *>grim* (black lines) and *PG>* (grey lines) for fed (full lines and circles) and starved (dashed lines and triangles). Both *>grim* and *PG>* starved animals have the same senseless stages.

Table 3.3: Comparisons in the response of Achaete stage to time and ecdysone, for male, or time, ecdysone and food, for female PGX larvae.

| Response: Achaete Stage | Sums of Squares | Df | F-value |
|----------------------------|--------------------|----------|-------------------|
| Females | | | |
| Time | 792.63 | 1 | 1256.41*** |
| Ecdysone | 172.24 | 1 | 273.02*** |
| Diet | 15.04 | 1 | 23.84*** |
| Time*Ecdysone | 173.05 | 1 | 274.30*** |
| Time*Diet | 1.34 | 1 | 2.12 |
| Ecdysone*Diet | 3.94 | 1 | 6.24* |
| Time* Ecdysone*Diet | 5.73 | 1 | 9.08** |
| Residuals | 566.52 | 898 | |
| Males | | | |
| Time | 161.61 | 1 | 311.85*** |
| Ecdysone | 34.62 | 1 | 66.80*** |
| Time*Ecdysone | 0.40 | 1 | 0.77 |
| Residuals | 60.63 | 117 | |

Females: $F_{7,898}=261.6$, p value<0.001, Adjusted $R^2=0.67$

Males: $F_{3,117}=129.5$, p value<0.001, Adjusted $R^2=0.76$

Significance code: * ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.001 , significant parameters are highlighted in bold.

Table 3.4: Comparisons in the response of Senseless stage to time and ecdysone, for male, or time, ecdysone and food, for female PGX larvae.

| Response: Senseless Stage | Sums of Squares | Df | F-value |
|------------------------------|--------------------|----------|------------------|
| Females | | | |
| Time | 166.28 | 1 | 692.59*** |
| Ecdysone | 66.52 | 1 | 277.06*** |
| Diet | 0.16 | 1 | 0.68 |
| Time*Ecdysone | 83.31 | 1 | 342.82*** |
| Time*Diet | 0.17 | 1 | 0.40 |
| Ecdysone*Diet | 3.84 | 1 | 13.98*** |
| Time* Ecdysone*Diet | 3.73 | 1 | 15.55*** |
| Residuals | 215.60 | 898 | |
| Males | | | |
| Time | 75.77 | 1 | 230.14*** |
| Ecdysone | 39.73 | 1 | 120.68*** |
| Time*Ecdysone | 16.53 | 1 | 50.22*** |
| Residuals | 38.52 | 117 | |

Females: $F_{7,898}=191.1$, p value<0.001, Adjusted $R^2=0.58$

Males: $F_{3,117}=137.2$, p value<0.001, Adjusted $R^2=0.77$

Significance code: * ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.001 , significant parameters are highlighted in bold.

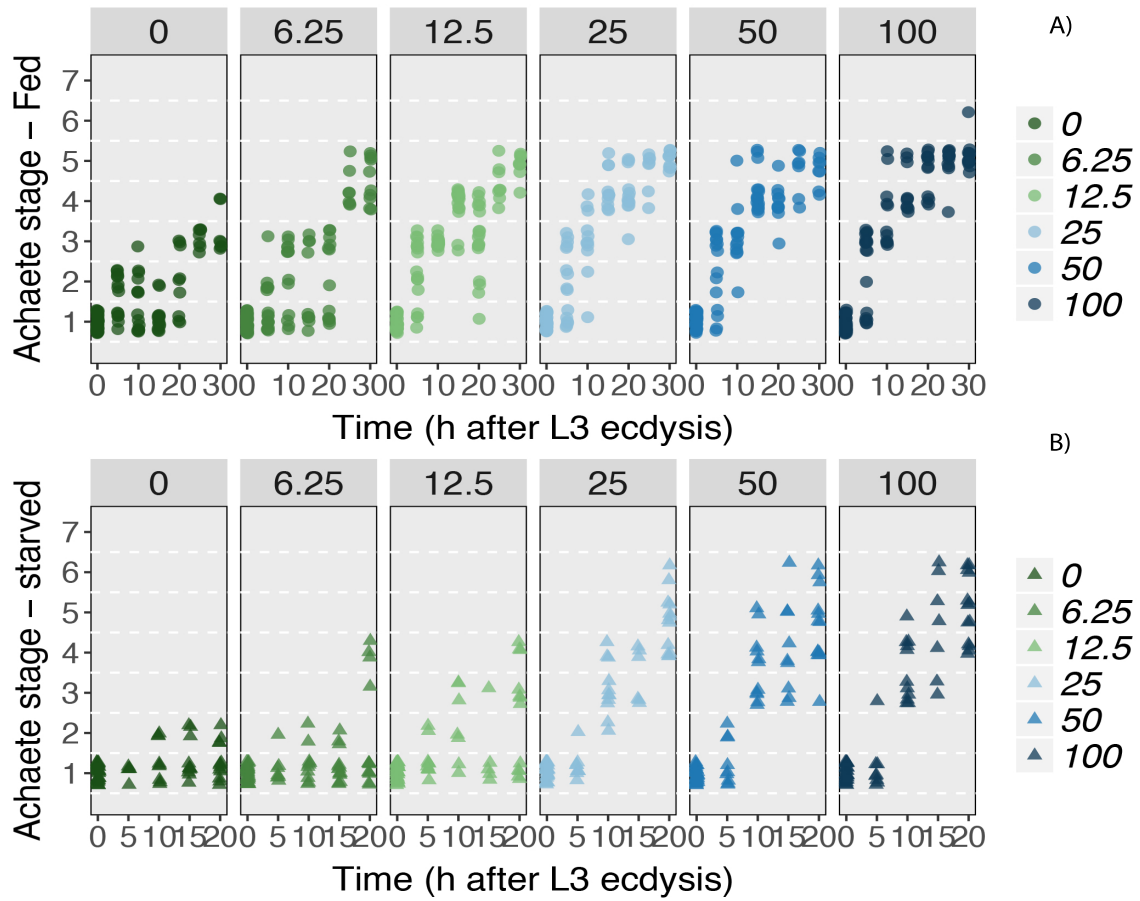


Fig. 3.4: The effects of ecdysone concentration on Achaete patterning rates in the wing discs fed and starved PGX larvae. (A) Achaete stage over time for fed PGX females. (B) Achaete stage over time for starved PGX females. Numbers above the graphs and in the figure legends refer to ecdysone concentrations (pg/mg of diet).

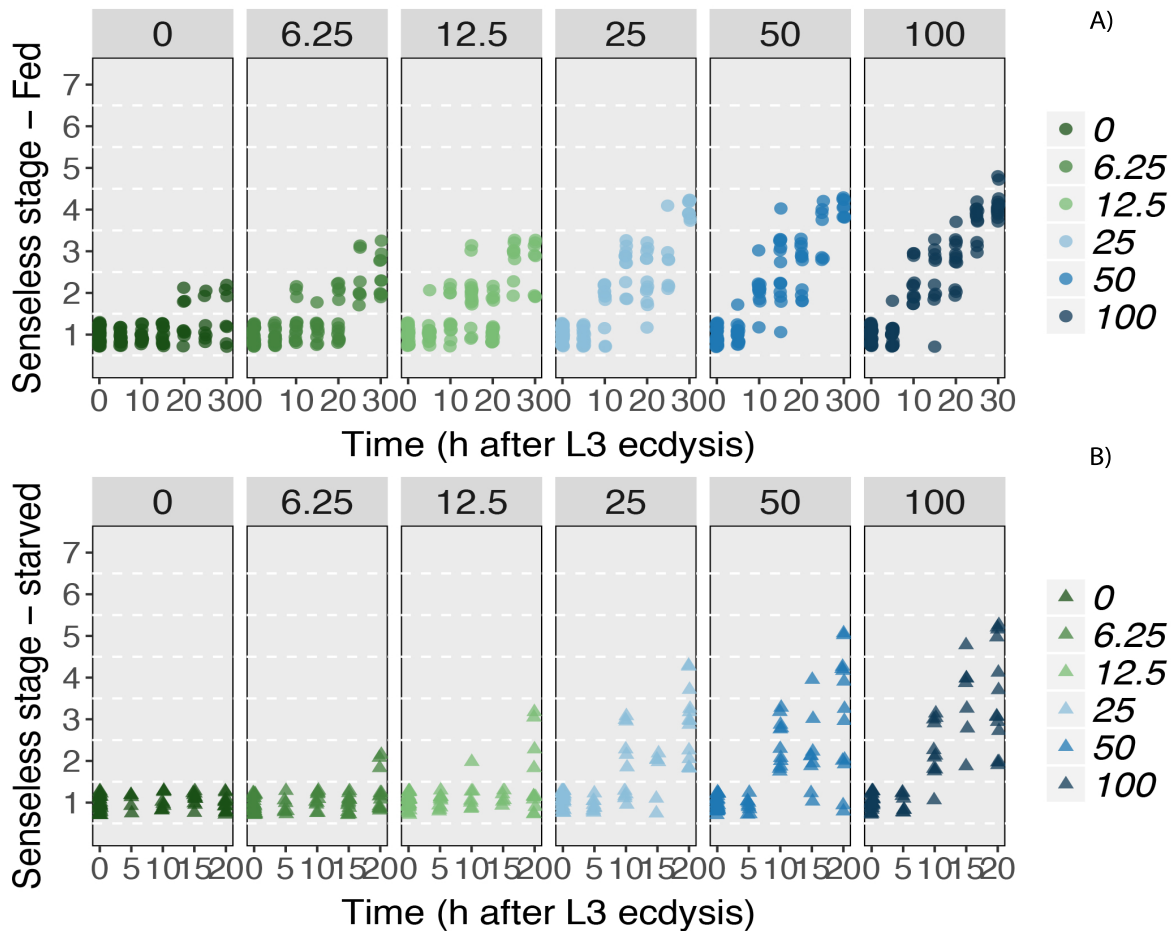


Fig.3.5: The effect of ecdysone concentration on Senseless patterning rates in the wing discs of fed and starved PGX females. (A) Senseless stage over time for fed female PGX larvae. (B) Senseless stage over time for starved PGX females. Numbers above the graphs and in the figure legends refer to ecdysone concentrations (pg/mg of diet).

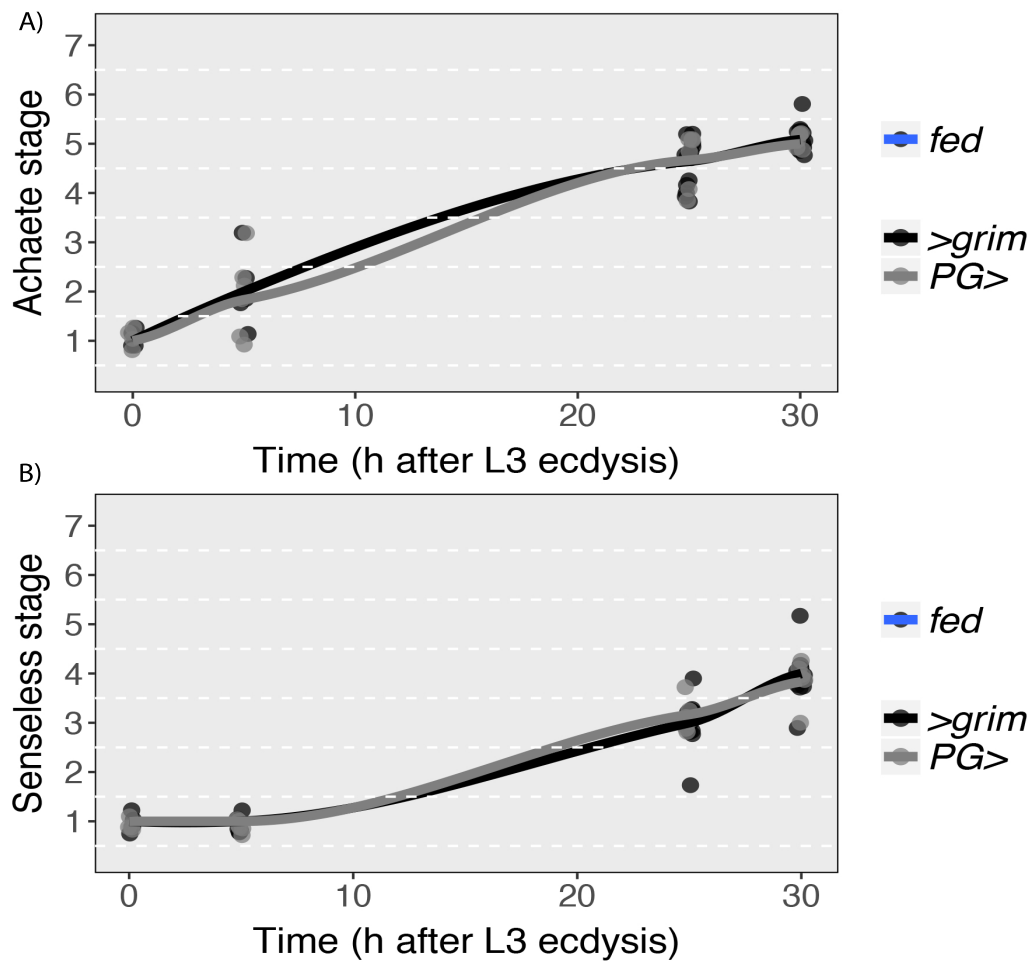


Fig.3.6: Achaete and Senseless patterning rates for male control larvae. (A) Achaete stage over time for *>grim* (black line) and *PG>* (grey line) males. (B) Senseless stage over time for *>grim* (black line) and *PG>* (grey line) males.

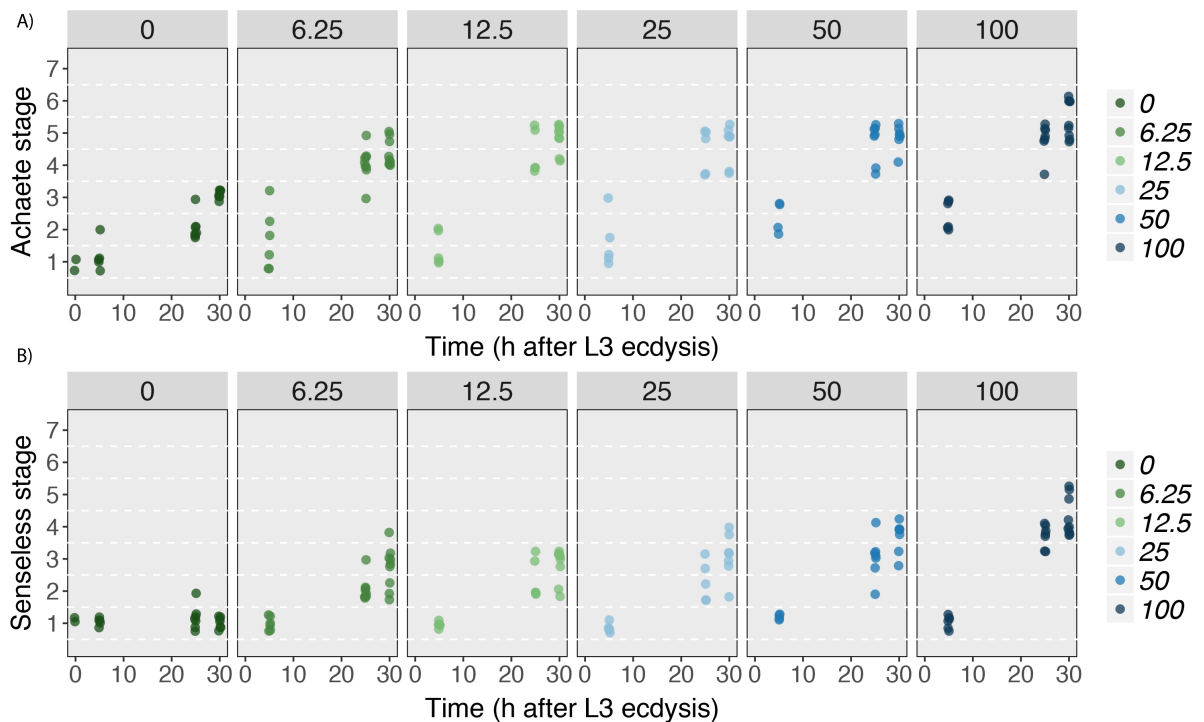


Fig.3.7: The effect of ecdysone concentration on Achaete and Senseless patterning rates in the wing discs of PGX males. (A) Achaete stage over time for PGX males. (B) Senseless stage over time for PGX males. Numbers above the graphs and in the figure legends refer to ecdysone concentrations (ng/mg of diet).

Testing the rate model versus the threshold model for wing disc patterning and growth

We hypothesized that variation in rates of patterning could arise as a result of differences in how growth or patterning responded to ecdysone. We thus proposed that growth or patterning could respond linearly to ecdysone concentrations, where ecdysone would alter the rates of these relationships, or that growth or patterning could respond to threshold levels of ecdysone, with changing concentrations of ecdysone controlling either exponential rate of increase or the timing of the threshold. Linear rate responses would indicate that ecdysone was responsible increasing or decreasing the rates of developmental processes, but not for turning them on or off. Threshold responses, on the other hand, are the mechanisms that turn developmental programs on or off, and these switch-like responses are known to induce cell fate transitions in a multitude of different contexts using mechanisms such as bi-stability, cooperativity, or even titration switches (Perrimon et al., 2012).

Testing between these two models requires fitting our ecdysone dose response data from PGX larvae with either linear models or a threshold-based model like the Hill's function. Because our data sets were more complete for female larvae, we only tested between linear and threshold models for females. The linear model fits data to the following equation:

$$y = ax + b$$

Equation 1.1: Linear model function

In this model, the parameters (a) and (b) represent the slope of the line and the value where the line would cross the y axis, respectively. In all cases, x = time (h after the third instar moult) and y = log₁₀ disc area, Achaete stage, or Senseless stage. If a linear model gives the best fit, then (a) represents the growth or patterning rate.

The Hill's equation is a type of four-parameter logistic equation typically used to describe threshold responses. For the Hill's function, (a) is the lower asymptote, (b) is the exponential slope of the curve, (c) is the midpoint between the two asymptotes and (d) is the upper asymptote.

$$y = d + \frac{(a - d)}{(1 + (\frac{x}{c})^b)}$$

Equation 1.2: Hill's function model

We could not fit the Hill's function to the disc size data from either starved or fed PGX larvae at any ecdysone concentration, suggesting that threshold models are inappropriate for disc growth rates. This supports our hypothesis that wing disc size responds to variation in ecdysone concentrations by adjusting their growth rates (also see Table 3.1).

In contrast, we could fit Hill's functions to the Achaete and Senseless patterning stage data for all concentrations of ecdysone, and for both starved and fed PGX larvae. We next asked whether the Hill's function improved the fit to the data over linear models, comparing both models using ANOVA. When compared to the linear models, the Hill's function significantly improved the fit to the relationship between Achaete stage and time at ecdysone concentrations ≥ 6.25 ng/mg for fed larvae and at ≥ 25 ng/mg for starved larvae (Table 3.5). For Senseless, the Hill's function significantly improved the fit between Senseless stage and time at ecdysone concentrations ≥ 25 ng/mg in both starved and fed larvae when compared to the linear models. Taken together, our data support the

hypothesis that patterning gene stage is regulated by threshold responses to ecdysone. Variations in initial ecdysone concentrations will create different patterning trajectories from early stages.

Table 3.5: Analysis of Variance Table (ANOVA) from the comparison between models, for fed and starved PGX larvae, comparing the fit of linear models versus Hill's function models for the response of Achaete stage (top) and Senseless stage (bottom) to time for a range of ecdysone concentrations.

| Response: Patterning Gene Stage | Sums of Squares | Df | F-value |
|--|--------------------|-----------|-----------------|
| Achaete | | | |
| Linear versus Threshold– Fed (ecdysone ≥ 6.25) | -30.32 | -2 | 24.98*** |
| Linear versus Threshold – Starved (ecdysone ≥ 6.25) | -6.59 | -2 | 2.94 |
| Linear versus Threshold – Fed (ecdysone ≥ 25) | -51.15 | -2 | 83.98*** |
| Linear versus Threshold – Starved (ecdysone ≥ 25) | -19.58 | -2 | 25.51*** |
| Senseless | | | |
| Linear versus Threshold – Fed (ecdysone ≥ 6.25) | Does not resolve | | |
| Linear versus Threshold – Starved (ecdysone ≥ 6.25) | -1.76 | -2 | 1.52 |
| Linear versus Threshold – Fed (ecdysone ≥ 25) | -2.41 | -2 | 5.78** |
| Linear versus Threshold – Starved (ecdysone ≥ 25) | -3.81 | -2 | 3.90* |

3.2 – Exploring the relationship between ecdysone concentration, wing disc growth and wing disc patterning in late third instar stages

The second aim of our study was to understand how the wing disc compensates for delays in patterning induced in the earlier phases of the third instar, so that all discs reached the same final pattern at pupariation. To address this question, we explored how ecdysone concentrations affected the rate of patterning in the second half of the third instar.

In our first experiment, we determined whether ecdysone was required for disc growth and patterning in later phases of the third instar. We collected animals at the moult to third instar, reared them to either 28 or 55 h AL3E at 17°C, and then shifted them to 29°C to ablate their prothoracic gland (PGX). We selected these two times in an attempt to choose a stage that would be as close as possible to pupariation, the development stage where patterning is known to be invariant across all discs (Oliveira et al., 2014), but where ecdysone still affected patterning. For this experiment, the control genotype was *>grim*, and these larvae were treated in the same manner as the PGX larvae.

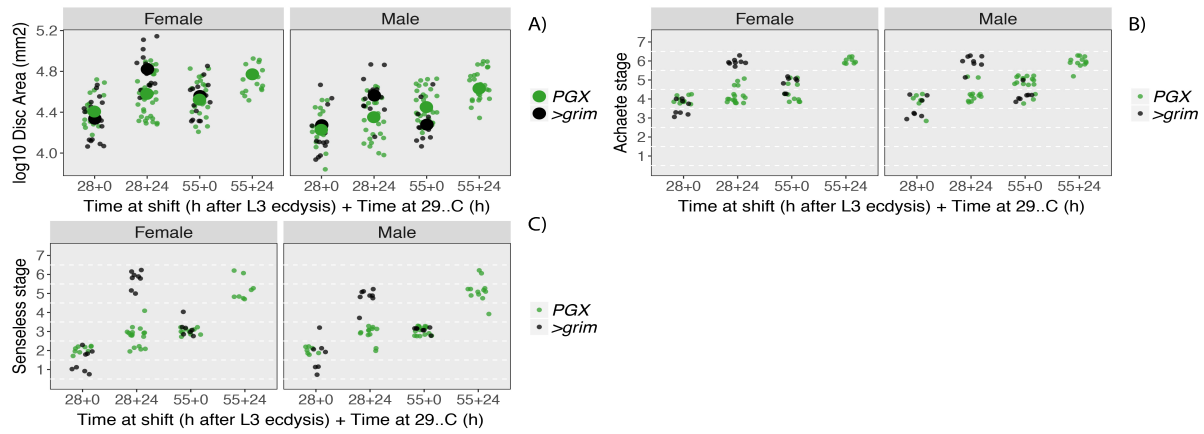


Fig.3.8: Growth and patterning in the wing discs after prothoracic gland ablations (PGX) at 28 and 55 h AL3E compared to >grim larvae. (A) Disc area (log₁₀) over time for PGX (green circles) and >grim (black circles) larvae. (B) Achaete stage over time for PGX (green circles) and >grim (black circles) larvae. (C) Senseless stage over time for PGX (green circles) and >grim (black circles) larvae. All >grim larvae pupariated between 55+16 and 55+24 hours AL3E.

Both genotypes were reared on standard food for an additional 24 h after the shift to 29°C, after which their wing discs were collected and processed for immunocytochemistry.

While the PGX and >grim larvae had wing discs of indistinguishable sizes at the onset of the shift at 28 h AL3E (both females and males), 24 h after the shift PGX larvae had significantly smaller discs than the controls (Fig. 3.8A; Table 3.6). For the shift at 55 h AL3E, PGX males had larger wing discs than controls at 55 h AL3E, although female wing discs were indistinguishable in size between genotypes. All control larvae had pupariated between 55+16h and 55+24h AL3E in this experiment. The PGX larvae failed to pupariate during this interval. Because of the difference in life stage, we could not determine whether the growth in the wing discs was impaired in PGX larvae relative to the controls. Using an ANOVA to compare growth rates of wing discs, we observed that time and genotype, time and sex, and time, sex and genotype have significant interactions (Table 3.6).

Table 3.6: Comparisons in the response of log₁₀ disc area to time, sex and genotype after prothoracic gland ablation at either 28 or 55h AL3E.

| Response: log ₁₀ disc area | Sums of Squares | Df | F-value |
|--|--------------------|----------|------------------|
| Time | 4.44 | 3 | 285.07*** |
| Sex | 1.87 | 1 | 361.32*** |
| Genotype | 0.19 | 1 | 36.49*** |
| Time*Sex | 0.23 | 3 | 14.66*** |
| Time*Genotype | 0.99 | 2 | 95.52*** |
| Sex*Genotype | 0.003 | 1 | 0.45 |
| Time*Sex*Genotype | 0.15 | 2 | 14.29*** |
| Residuals | 1.35 | 260 | |

$F_{13, 260}=107.5$, p value<0.001, Adjusted $R^2=0.84$

Significance code: *≤0.05, **≤0.01, ***≤0.001, significant parameters are highlighted in bold.

At the onset of the shift at 28 h AL3E, Achaete and Senseless stages were indistinguishable between PGX larvae and controls. However, 24 hours after the shift, all PGX larvae showed delayed pattern for both patterning gene products relative to controls. For the shift at 55 h AL3E, male PGX larvae were more advanced in their Achaete stage than controls. Female PGX larvae showed indistinguishable Achaete stages when compared to controls, and both male and female PGX larvae overlapped in their Senseless stages when compared to controls. Although we could not compare PGX larvae directly to controls 24 h after the shift, since control larvae had pupariated, PGX larval

discs had not yet reached their final pattern (stage 7) for either Achaete or Senseless, suggesting their patterning was delayed. When comparing patterning stage between sexes and genotypes we found significant interactions between time and genotype, sex and genotype and time, sex and genotype (Table 3.7).

Table 3.7: Comparisons in the response of Achaete or Senseless stage to time, sex and genotype after prothoracic gland ablation at either 28 or 55h AL3E.

| Response: Achaete | Sums of Squares | Df | F-value |
|--------------------------|--------------------|----------|------------------|
| Time | 67.44 | 3 | 150.62*** |
| Sex | 0.05 | 1 | 0.57 |
| Genotype | 5.69 | 1 | 38.12*** |
| Time*Sex | 0.32 | 3 | 0.54 |
| Time*Genotype | 27.10 | 2 | 90.78*** |
| Sex*Genotype | 0.81 | 1 | 5.40* |
| Time*Sex*Genotype | 1.58 | 2 | 5.30** |
| Residuals | 18.36 | 123 | |
| Response: Senseless | Sums of Squares | Df | F-value |
| Time | 166.21 | 3 | 344.12*** |
| Sex | 0.37 | 1 | 2.29 |
| Genotype | 20.42 | 1 | 126.83*** |
| Time*Sex | 0.95 | 3 | 1.97 |
| Time*Genotype | 46.88 | 2 | 145.58*** |
| Sex*Genotype | 0.80 | 1 | 4.96* |
| Time*Sex*Genotype | 1.61 | 2 | 4.99** |
| Residuals | 19.80 | 123 | |

Achaete: $F_{13, 123}=50.78$, p value<0.001, Adjusted $R^2=0.83$

Senseless: Achaete: $F_{13, 123}=104$, p value<0.001, Adjusted $R^2=0.91$

Significance code: * ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.001 , significant parameters are highlighted in bold.

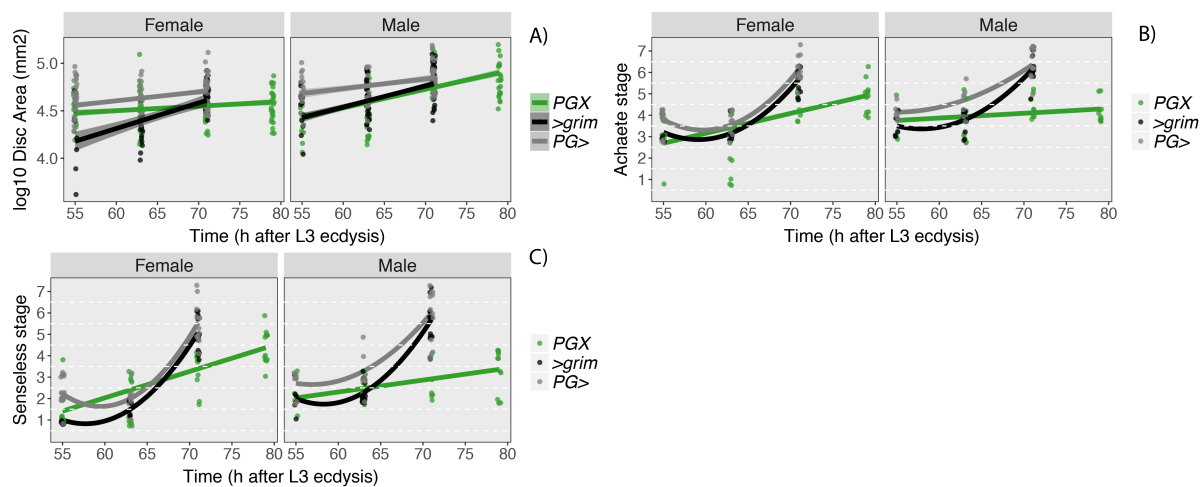


Fig.3.9: Comparing the growth and patterning of the wing discs in prothoracic gland ablations (PGX) at 55 h AL3E to control genotypes. (A) Disc area (log₁₀) for PGX (green), >grim (black) and PG> (grey) female and male larvae. (B) Achaete stage over time in PGX (green), >grim (black) and PG> (grey) female and male larvae. (C) Senseless patterning rates for PGX (green), >grim (black) and PG> (grey) genotypes. All control larvae pupariated between 55+16 and 55+24 hours.

Oliveira et al (2014) had previously shown that discs coordinate their patterning at pupariation. Because all control animals pupariated within 24 h after the shift to 29°C at 55 h AL3E, we chose this time point for further study. In this experiment, we compared larvae from both control genotypes, *>grim* and *PG>*, to larvae from our experimental genotype, PGX. In this experiment third instar larvae were shifted to 29°C at 55 h AL3E, sexed, and fed on standard food. Wing discs were collected at four different time points for the PGX larvae, 55+0 h, 55+8 h, 55+16 h and 55+24 h. Control larvae were sampled at the first three time points. All control larvae had pupariated by 55+24 h.

Table 3.8: Comparisons in the response of \log_{10} disc area to time, sex and genotype for prothoracic gland ablations at 55h AL3E without ecdysone supplement.

| Response: \log_{10} disc area | Sums of Squares | Df | F-value |
|------------------------------------|--------------------|----------|------------------|
| Time | 3.99 | 1 | 339.43*** |
| Sex | 2.48 | 1 | 211.00*** |
| Genotype | 1.96 | 2 | 83.14*** |
| Time*Sex | 0.31 | 1 | 26.75*** |
| Time*Genotype | 0.57 | 2 | 24.10*** |
| Sex*Genotype | 0.10 | 2 | 4.12* |
| Time*Sex*Genotype | 0.37 | 2 | 15.87*** |
| Residuals | 4.73 | 402 | |

$F_{11,402}=67.96$, p value<0.001, Adjusted $R^2=0.64$

Significance code: * ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.001 , significant parameters are highlighted in bold.

Table 3.9: Comparisons in the response of Achaete or Senseless stage to time, sex and genotype for prothoracic gland ablations at 55h AL3E without ecdysone supplement.

| Response: Achaete | Sums of Squares | Df | F-value |
|--------------------------|--------------------|----------|------------------|
| Time | 67.44 | 3 | 150.62*** |
| Sex | 0.05 | 1 | 0.57 |
| Genotype | 5.69 | 1 | 38.12*** |
| Time*Sex | 0.32 | 3 | 0.54 |
| Time*Genotype | 27.10 | 2 | 90.78*** |
| Sex*Genotype | 0.81 | 1 | 5.40* |
| Time*Sex*Genotype | 1.58 | 2 | 5.30** |
| Residuals | 18.36 | 123 | |
| Response: Senseless | Sums of Squares | Df | F-value |
| Time | 166.21 | 3 | 344.12*** |
| Sex | 0.37 | 1 | 2.29 |
| Genotype | 20.42 | 1 | 126.83*** |
| Time*Sex | 0.95 | 3 | 1.97 |
| Time*Genotype | 46.88 | 2 | 145.58*** |
| Sex*Genotype | 0.80 | 1 | 4.96* |
| Time*Sex*Genotype | 1.61 | 2 | 4.99** |
| Residuals | 19.80 | 123 | |

Achaete: $F_{13,123}=50.78$, p value<0.001, Adjusted $R^2=0.83$

Senseless: $F_{13,123}=104$, p value<0.001, Adjusted $R^2=0.91$

Significance code: * ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.001 , significant parameters are highlighted in bold.

Wing discs growth rates were linear on a \log_{10} scale for all three genotypes. Comparing the growth rate of wing discs across genotypes and sexes, we found significant interactions between time and sex, time and genotype, sex and genotype, and time, sex, and genotype (Table 3.8). Despite these differences, the growth rates of wing discs of PGX larvae did not differ significantly from both controls. This suggests that the differences in growth rates between genotypes were independent of ecdysone concentrations.

For patterning progression, PGX larvae showed linear patterning rates for both Achaete and Senseless. In contrast, both control genotypes demonstrated exponential rates of patterning for both gene products. Using ANOVAs to model the effects of genotype, sex, and time on patterning stage, we found that for both Achaete and Senseless stage, the interaction between time and genotype, sex

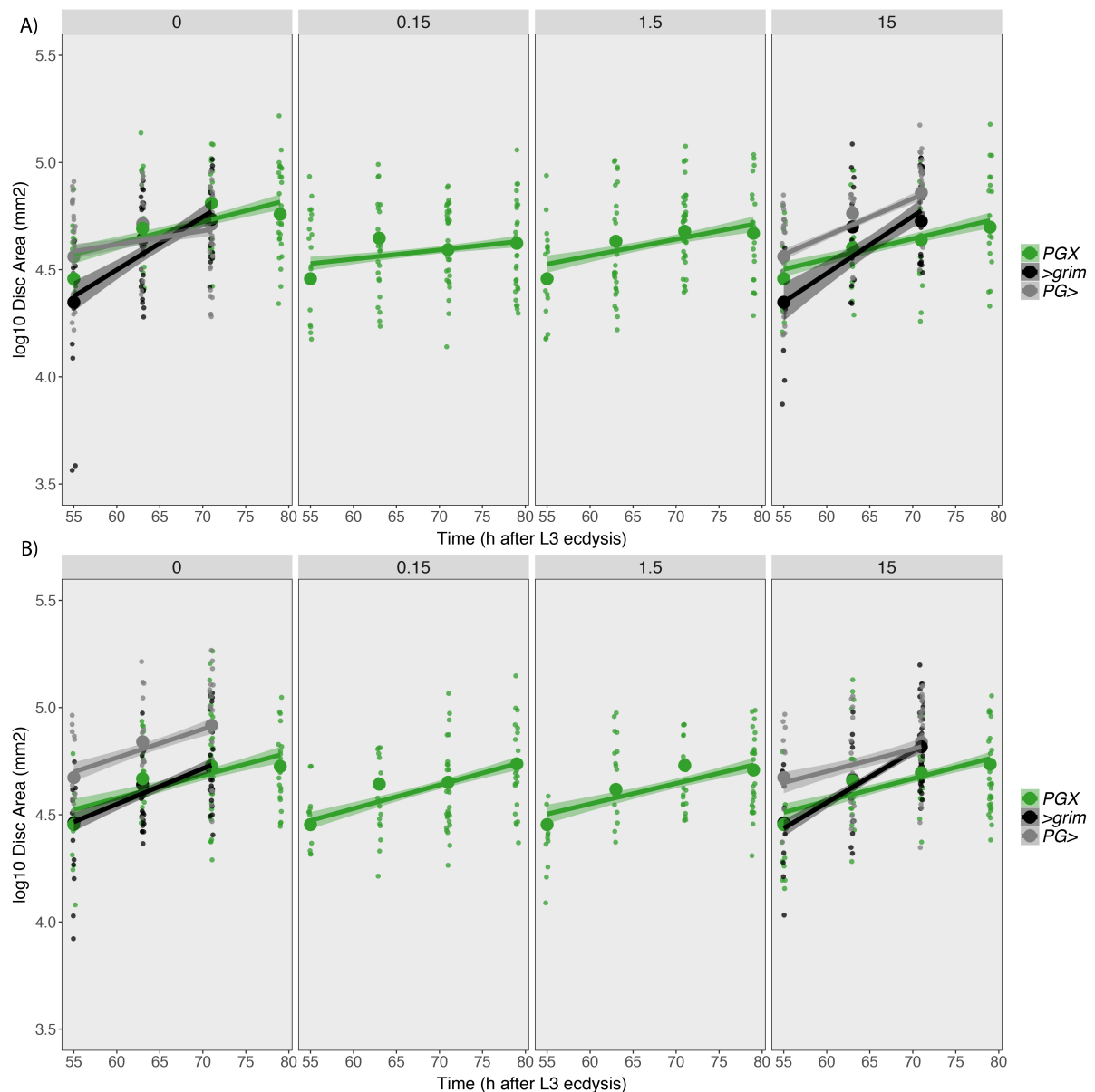


Fig.3.10: Growth rates in the wing discs after prothoracic gland ablation (PGX) at 55h AL3E and controls, *>grim* and *PG>*. (A) Disc area (\log_{10}) over time for PGX (green), *>grim* (black) and *PG>* (grey) female larvae. (B) Disc area (\log_{10}) over time for PGX (green), *>grim* (black) and *PG>* (grey) male larvae. Numbers above the graphs refer to ecdysone concentration (pg/mg of diet).

and genotype, and time, sex and genotype were significant (Table 3.9). Thus, although ecdysone does not appear to significantly affect wing disc growth at this stage, it did continue to control the rate of wing discs patterning.

For the final experiment, our aim was to determine the effect of ecdysone on growth and patterning of the wing discs at later stages of development as a means of understanding whether and how this hormone might act to coordinate pattern at pupariation. Again, we predicted that ecdysone could coordinate patterning by regulating their linear rates or by regulating a threshold response. If ecdysone regulated patterning by regulating its rates of progression, a linear model would fit best:

Table 3.10: Comparisons in the response of \log_{10} disc area to time, ecdysone and genotype both for females (top) and males (bottom) with 0 and 15ng/mg of ecdysone after prothoracic gland ablation at 55h AL3E.

| Response: \log_{10} disc area | Sums of Squares | Df | F-value |
|------------------------------------|--------------------|----------|------------------|
| Females | | | |
| Time | 4.17 | 1 | 327.66*** |
| Ecdysone | 0.00 | 1 | 0.01 |
| Genotype | 0.44 | 2 | 17.18*** |
| Time*Ecdysone | 0.07 | 1 | 5.66* |
| Time*Genotype | 0.76 | 2 | 29.67*** |
| Ecdysone*Genotype | 0.52 | 2 | 20.55*** |
| Time*Ecdysone*Genotype | 0.17 | 2 | 6.66** |
| Residuals | 5.30 | 416 | |
| Males | | | |
| Time | 3.54 | 1 | 370.39*** |
| Ecdysone | 0.02 | 1 | 2.13 |
| Genotype | 2.15 | 2 | 112.52*** |
| Time*Ecdysone | 0.00 | 1 | 0.42 |
| Time*Genotype | 0.32 | 2 | 16.94*** |
| Ecdysone*Genotype | 0.21 | 2 | 10.96*** |
| Time*Ecdysone*Genotype | 0.07 | 2 | 3.49* |
| Residuals | 3.76 | 395 | |

$F_{11,552}=66.24$ p value<0.001, Adjusted $R^2=0.56$

Significance code: * ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.001 , significant parameters are highlighted in bold.

$$y = ax + b$$

Equation 1.3: Linear model function

Because patterning terminates at pupariation, when discs reach their final patterning stage, threshold responses would not fit functions with clear upper asymptotes like the Hill's function. Instead, we predict that threshold responses would be best modelled using a quadratic function:

$$y = a + bx + cx^2$$

Equation 1.4: Quadratic model function

We selected female and male larvae from the three genotypes, PGX, >grim, and PG>, and fed them standard food with four different ecdysone concentrations (0, 0.15, 1.5, 15 ng/mg of diet) for the PGX larvae, or only two ecdysone concentrations (0 and 15 ng/mg of diet) for the >grim and

PG>. These concentrations were chosen as previous experiments by Dr. Marisa Oliveira had shown that *PGX* larvae pupariate precociously at higher ecdysone concentrations. We sampled the wing discs at either three (control) or four (*PGX*) time points (55+0, 55+8, 55+16, 55+24h AL3E).

When examining growth, we observe that the wing discs of the two control genotypes behave similarly to the previous experiment, growing linearly on a \log_{10} scale throughout time (Fig.3.9).

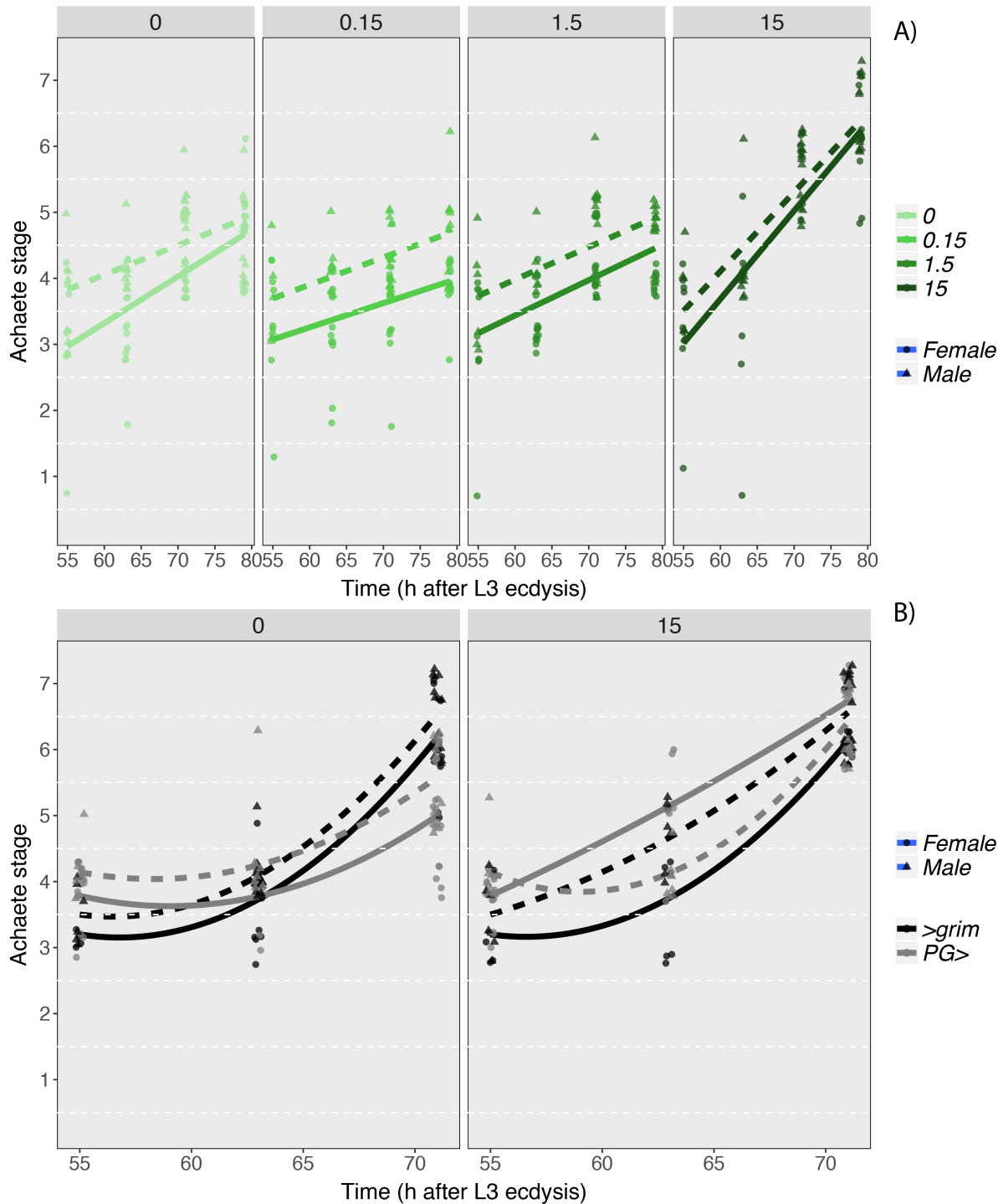


Fig.3.11: Achaete patterning rates after prothoracic gland ablation (PGX) at 55 h AL3E and in control larvae, *>grim* and *PG>*. (A) Achaete stage over time in *PGX* female (solid line and circles) and male (dashed line and triangles) larvae. (B) Achaete stage over time in *>grim* (black) and *PG>* (grey) female (solid lines and circles) and male (dashed lines and triangles) larvae. Numbers above the graphs refer to ecdysone concentrations (pg/mg of diet).

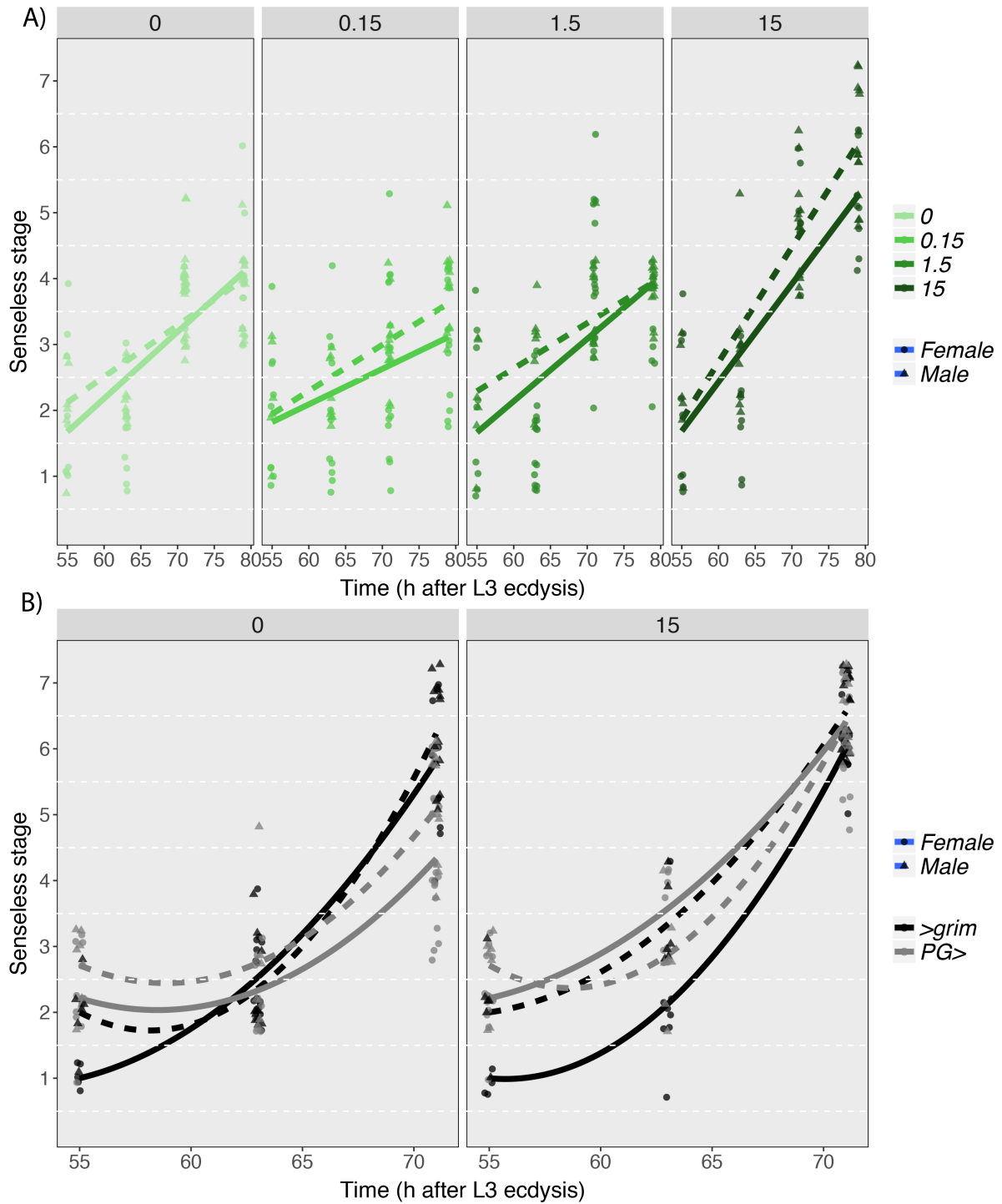


Fig.3.12: Senseless patterning rates in prothoracic gland ablations (PGX) at 55 h AL3E and controls, >grim and PG> larvae. (A) Senseless stage over time in PGX female (solid line and circles) and male (dashed line and triangles) larvae. (B) Senseless stage over time in >grim (black) and PG> (grey) female (solid lines and circles) and male (dashed lines and triangles) larvae. Numbers above the graphs refer to ecdysone concentrations (pg/mg of diet).

Adding ecdysone to the food did not influence the time to pupariate (Fig.3.10), although it did increase the growth rate in wing discs from PG> female and >grim male larvae. We did not observe any effects of ecdysone on the growth rate of the wing discs in PGX, PG> male, or >grim female larvae. For female control larvae, we observed significant interactions between ecdysone and time, ecdysone and genotype, time and genotype, and time, ecdysone, and genotype (Table 3.10). For males, ecdysone and genotype, time and genotype, and time, ecdysone, and genotype show significant interactions with wing disc size. These significant interactions result from the sex and genotype-specific effects of ecdysone on growth in control genotypes.

Regarding patterning, we observed that without ecdysone both males and females demonstrated exponential patterning rates for Achaete. Adding 15 ng/mg ecdysone to the food changed the response *PG>* female larvae and *>grim* male larvae, which both exhibited linear relationships between Achaete stage and time. When fed standard food with 15 ng/mg of ecdysone, Achaete retained exponential responses over time in *PG>* male larvae and *>grim* female larvae (Fig.3.11). For Senseless patterning, both with and without ecdysone, all sexes and control genotypes maintained exponential patterning rates (Fig.3.12).

In the wing discs of PGX larvae, Achaete patterning rates increased with increasing ecdysone concentration in both females and males (Fig.3.11). The only significant interaction for Achaete stage is time and ecdysone; there do not appear to be any sex-specific effects of ecdysone on Achaete patterning rates in PGX larvae. Furthermore, only the highest dose of ecdysone (15 ng/mg) significantly increased Achaete patterning rates. Similarly, for Senseless ecdysone increases the patterning rate for both sexes in PGX larvae (Fig.3.12), with a significant interaction between time and ecdysone (Table 3.11). In addition, only the highest concentration of ecdysone significantly increased Senseless patterning rates. Given that we only see significant effects on patterning rates at the highest ecdysone concentrations, and that we did not test the effects of ecdysone at concentrations higher than 15 ng/mg, it is difficult to discern whether ecdysone affects patterning rates in a linear or an exponential manner.

Table 3.11: Comparisons in the response of Achaete (top) or Senseless (bottom) stage to time, ecdysone and sex after prothoracic gland ablation at 55h AL3E.

| Response: Achaete | Sums of Squares | Df | F-value |
|--------------------------------|----------------------------|-----------|------------------|
| Time | 114.88 | 1 | 255.07*** |
| Ecdysone | 46.39 | 1 | 103.00*** |
| Sex | 22.98 | 1 | 51.03*** |
| Time*Ecdysone | 26.69 | 1 | 59.25*** |
| Time*Sex | 0.56 | 1 | 0.27 |
| Ecdysone*Sex | 1.13 | 1 | 0.11 |
| Time*Ecdysone*Sex | 0.03 | 2 | 0.81 |
| Residuals | 154.47 | 343 | |
| Response: Senseless | Sums of Squares | Df | F-value |
| Time | 239.47 | 1 | 294.70*** |
| Ecdysone | 63.54 | 1 | 78.19*** |
| Sex | 8.55 | 1 | 10.52** |
| Time*Ecdysone | 31.95 | 1 | 39.32*** |
| Time*Sex | 0.01 | 1 | 0.016 |
| Ecdysone*Sex | 0.92 | 1 | 1.14 |
| Time*Ecdysone*Sex | 1.31 | 1 | 1.61 |
| Residuals | 278.72 | 343 | |

Achaete: $F_{7,343}=72.44$ p value<0.001, Adjusted $R^2=0.59$

Senseless: $F_{7,343}=64.22$, p value<0.001, Adjusted $R^2=0.56$

Significance code: * ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.001 , significant parameters are highlighted in bold.

4. Discussion

Think left and think right and think low and think high.
Oh, the thinks you can think up if only you try! – Dr. Seuss

Ensuring that specific traits have enough plasticity to adjust to different environments, while other traits bear sufficient robustness to ensure organ function is critical to fitness in all organisms. How these two features are coordinated to create fully functioning organs and organisms is still unknown. The wings of *D. melanogaster* are good examples of how plastic and robust developmental processes can be coordinated, since wings can vary in size but maintain the same pattern of the veins and bristles. By manipulating growth rates, developmental timing, and systemic ecdysone concentrations, we have observed how each affects the size of wing imaginal discs and their rates of patterning. These studies will provide insight into how organ development coordinates plastic with robust developmental processes, thus giving rise to functional organisms with traits appropriate for their developmental environments.

As a premise to the current study, Oliveira et al. (2014) observed that even though the final pattern of wing discs is invariant, the rates of patterning are plastic in the early to middle stages of development. How variation in patterning rates and final robustness of pattern are integrated to create a fully functioning organ is still unknown, but our study has shed light on how plasticity and robustness are coordinated in the wing disc.

4.1 – Exploring the effects of ecdysone concentration in wing disc growth and wing disc patterning in early third instar stages

Plasticity in morphological phenotypes is generated primarily through alterations during development. In social insects, castes are determined in the larval stages through nutritional differences (Huang et al., 2013), although the full phenotypes will manifest only in the adult stage. Also in *D. melanogaster*, the effects of the developmental environment alter phenotypes primarily during the larval stages. In this species, and many other insects, changes in the growth of the body and organs in response to the larval environment generates much of the observed variation in body size and shape between individuals.

D. melanogaster larvae rely on the insulin/TOR pathway to promote growth in response to nutritional conditions (Nässel et al., 2015). The insulin/TOR pathway is stimulated by several peptides called the *Drosophila* insulin like peptides (dILPs) (Kannan & Fridell, 2013). When larvae are deprived of amino acids, the level of circulating dILPs decreases, and causes a concomitant decrease in the activity of the insulin/TOR pathway (Ikeya et al 2002, Colombani et al 2003; Geminard et al 2009; Koyama & Mirth 2016; Delanoue et al 2016). Decreased insulin/TOR results in a low growth rates in the body and the imaginal tissues. Thus, tissues rely on the activity of insulin/TOR to tune their growth rates according to the developmental environment.

However, insulin/TOR is not the only regulator of growth, and there is mounting evidence that ecdysone plays a key role in mediating the effects of nutrition on body and organ growth (Shingleton et al., 2008). We find that when larvae are starved of yeast, ecdysone can rescue the repressed growth in the wings. Previous studies suggest that ecdysone induces these effects by bypassing the insulin/TOR pathway; reducing ecdysone synthesis increases the mRNA expression of the negative regulator of insulin/TOR, 4E-BP (Herboso et al 2015). Precisely how ecdysone exerts these effects is as yet unknown. To fully understand how insulin/TOR and ecdysone interact to regulate growth in the wing discs would require manipulating both pathways in concert in this organ and examining the effects on their downstream regulators.

Further, how starved larvae sustain the growth of their wing discs, if given enough ecdysone, is unknown. The protein derived from yeast is essential for maintaining cell division and growth in

larvae (Britton and Edgar, 1998) Possibly, starved PGX larvae that receive ecdysone supplement reallocate resources like amino acids from other tissues to sustain the growth of their imaginal discs.

Although the wing discs of fed PGX females did not change their growth rates in response to ecdysone, the male wing discs did. Wing discs from male PGX larvae increased their growth rate in response to increasing ecdysone concentrations. The wing discs of male and female larvae have previously been shown to differ in their growth rates (Testa et al., 2013). Our results highlight that this difference may be underpinned by a difference in their response to key developmental hormones.

In terms of wing disc patterning, ecdysone plays an important role in both fed and starved PGX larvae. In fed larvae, Achaete stage showed some degree of progression in PGX larvae, whereas Senseless does not. Potentially, this could be because Achaete is more sensitive to ecdysone than Senseless. Although we assume that by ablating the prothoracic gland we eliminate ecdysone synthesis completely, we do not yet know how fast ecdysone titres degrade after this ablation. If Achaete is more sensitive to ecdysone, it is possible that the residual ecdysone in PGX larvae is sufficient to sustain some progression of Achaete pattern, but not Senseless.

In starved larvae, neither Achaete nor Senseless progress in pattern. The onset of Senseless patterning in the wing imaginal discs correlates with the first ecdysone peak at the third instar (Mirth et al 2009). This peak can shift in time depending on environmental conditions (Ghosh et al., 2013; Koyama et al., 2014), and in starved larvae this first ecdysone peak is strongly suppressed. Presumably, starved PGX larvae have even lower ecdysone titres than fed PGX larvae due to the combined action of the prothoracic gland ablation and the negative effects of starvation on ecdysone synthesis. This would result in greater suppression of patterning rates for Achaete and Senseless.

In addition to plasticity in the timing of the first ecdysone peak, if there is also plasticity in the maximum concentration of the ecdysone peak, this could cause additional variation in the relative timing of patterning in the wing discs. Our data demonstrates that the wing discs reach higher stages of both Achaete and Senseless at higher concentrations of ecdysone. Potentially, changes in the timing or concentration of the first ecdysone peak could give rise to the variation in the patterning trajectories observed in Oliveira et al., 2014.

Both Achaete and Senseless showed threshold responses to ecdysone, which can be modelled by a Hill's function. Threshold responses have been well studied in a number of scenarios, most notably bi-stable switches in embryonic segment formation in *D. melanogaster* and also cooperative switches such as sex differentiation (Wang & Bell, 1994). Having these types of responses suggests that patterning rate of the wing discs needs to be switched on within a narrow range of conditions, a feature typical of cell fate decisions and robust developmental responses. Further exploration of this data could give a stronger insight into how ecdysone affects this threshold response. Through mathematical modelling, we could identify if ecdysone is affecting the timing that patterning gets turned on, thereby inducing plasticity in patterning trajectories as seen in Oliveira et al. (2014). Ecdysone could also be affecting the exponential rate of increase in patterning, creating differences in how quick or slow the wing discs pattern. Finally, ecdysone could also be affecting the final stage of patterning within a given time interval.

4.2 – Exploring the relationship between ecdysone concentration, wing disc growth and wing disc patterning in late third instar stages

When development is reaching its final stages, it is important to ensure that all organs have the features necessary to function properly, including their structures and pattern. For example, even though the castes of social insects differ in their body sizes and shapes, all the members of the colony have the same number of tagma in their bodies: head, thorax, and abdomen (Huang et al., 2013). The same properties of robustness have been observed in *Drosophila* wing discs, where wing discs all reach the same patterning stage at the end of larval development (Oliveira et al., 2014). Furthermore, ecdysone is required throughout development to regulate this progression of patterning and also to regulate growth of wing discs (Mirth et al., 2009, Herboso et al., 2015). Our second set of experiments was designed to address how this coordination patterning with whole body developmental events at pupariation occurs and to identify the role ecdysone plays in regulating this coordination.

During the wandering stage, the larvae leave the food and search for a place to pupariate. This results in a number of physiological changes in the animal, as any remaining growth must occur in the absence of nutrition. In response to the small pulse of ecdysone that stimulates wandering, the larval fat body upregulates the production and secretion of a specific dILP, dILP6, which is thought to sustain the remaining growth of the imaginal tissues (Okamoto et al 2009; Slaidina et al 2009). Thus, it is perhaps unsurprising that we could not detect significant and consistent effects of ecdysone on the growth of the imaginal discs at late stages of third instar development. However, because our study did not compare the growth of starved versus fed wing discs at later stages we cannot rule out the possibility that ecdysone affects the growth of wing discs from starved larvae.

Our data regarding patterning during the later stages of the third instar was unable to resolve whether ecdysone controls patterning via rate or threshold mechanisms. The control larvae showed exponential rates of patterning for both Achaete and Senseless for the time interval sampled. In contrast, PGX larvae showed linear rates of increase. The fact that the PGX data does not resemble the controls at the highest ecdysone concentration might reflect the range of ecdysone concentrations chosen. We observed a switch point at 15 ng/mg of ecdysone in PGX larvae, the only ecdysone concentration that increased patterning rates. Increasing ecdysone concentrations beyond this concentration could trigger an exponential response in patterning that would allow the PGX larvae to reach the final patterning stages for Achaete and Senseless and to pupariate at a time similar to the controls.

Threshold responses are usually great mechanisms to create fast switches, and these responses can be sensitive to environmental conditions. Different environmental factors will cause variation in how patterning progresses to adjust to the larval conditions. But, in late larval development the wing imaginal discs need to reach the final patterning stage, so they can produce the correct structure with the correct pattern before differentiating into the adult wing. The fastest way to ensure that discs reach the correct pattern would be to create a threshold response in patterning during last ecdysone peak in the third larval instar.

4.3 - Future Directions

Our studies have provided some much needed insight into how organs coordinate plastic with robust developmental processes, and the role that ecdysone plays in this coordination. However, there is still much to learn. Examining how ecdysone affects growth in the intermediate stages of development would resolve some of the discrepancies between experimental results and published data. Both Herboso et al. (2015) and Mendes & Mirth (2016) found that ecdysone is important for the growth of imaginal tissues in fed larvae. Furthermore, Mendes & Mirth (2016) also demonstrates that ecdysone only affects growth of the ovaries in the mid to later stages of the third instar, after the first ecdysone pulse. Our data consistently shows the opposite, but this could be due to sampling very early stages of the third instar, where insulin/TOR plays the primary role in regulating growth, and very late stages, such as wandering, where growth has almost stopped. Our data from the 28 h shift suggests that ecdysone does affect wing disc growth at intermediate stages, and studies during this interval would refine our current data and demonstrate that ecdysone changes its involvement in wing disc growth after the first ecdysone pulse and again after the ecdysone pulse leading to wandering.

Ecdysone and the insulin/TOR pathways are tightly connected, and interact with each other several times throughout development, but little is understood on how that affects growth and patterning. In addition, little is known also about how these two pathways affect sexes differently. Our data demonstrates that ecdysone and insulin/TOR are not solely regulating just patterning or growth respectively, but that there are interactions between these pathways to the point where one pathway can bypass the effects of the other. Determining the nature of how these two pathways interact could be achieved by simultaneously manipulating the activity of both pathways in the wing disc and examining the effects on growth and patterning rates. Exploring the effects on growth and patterning in both sexes would provide insight into how these interactions change depending on the sex.

5. Conclusions

How did it get so late so soon? – Dr. Seuss

In this study, we found that ecdysone plays an important role in regulating both plastic and robust developmental responses in the wing disc of *D. melanogaster*. The same hormone can coordinate both plastic and robust developmental processes via differences in the nature of how ecdysone affects disc growth versus disc patterning. Early on in development, ecdysone controls the rates of growth in response to environmental conditions like nutrition to ensure that a range of wing sizes can be generated. In contrast, threshold responses to ecdysone switch patterning on, such that this process is induced over a wide range of conditions. Towards the end of the larval stage, ecdysone directs the final stages of patterning without further effects on growth. The extent to which single hormones can mediate both plastic and robust developmental processes is yet unknown, but our findings form a template for understanding how plasticity and robustness could be coordinated in other organs and other organisms. Further, these findings bring us one step further to understanding the mechanisms that regulate plasticity and robustness both at the trait level and at the level of developmental processes, to create fully functional organs.

6. Bibliography

The more that you read, the more things you will know.
The more that you learn, the more places you'll go.
— Dr. Seuss in *I Can Read With My Eyes Shut!*

- Barkoulas, M., van Zon, J.S., Milloz, J., van Oudenaarden, A. and Félix, M.A., 2013. Robustness and epistasis in the *C. elegans* vulval signaling network revealed by pathway dosage modulation. *Developmental cell*, 24(1), pp.64-75.
- Beadle, G.W., Tatum, E.L. and Clancy, C.W., 1938. Food level in relation to rate of development and eye pigmentation in *Drosophila melanogaster*. *The Biological Bulletin*, 75(3), pp.447-462.
- Brakefield, P.M., Pijpe, J. and Zwaan, B.J., 2007. Developmental plasticity and acclimation both contribute to adaptive responses to alternating seasons of plenty and of stress in *Bicyclus* butterflies. *Journal of biosciences*, 32(3), pp.465-475.
- Britton, J.S. and Edgar, B.A., 1998. Environmental control of the cell cycle in *Drosophila*: nutrition activates mitotic and endoreplicative cells by distinct mechanisms. *Development*, 125(11), pp.2149-2158.
- Colombani, J., Raisin, S., Pantalacci, S., Radimerski, T., Montagne, J. and Léopold, P., 2003. A nutrient sensor mechanism controls *Drosophila* growth. *Cell*, 114(6), pp.739-749.
- Delanoue, R., Meschi, E., Agrawal, N., Mauri, A., Tsatskis, Y., McNeill, H. and Léopold, P., 2016. *Drosophila* insulin release is triggered by adipose Stunted ligand to brain Methuselah receptor. *Science*, 353(6307), pp.1553-1556.
- Félix, M.A. and Wagner, A., 2008. Robustness and evolution: concepts, insights and challenges from a developmental model system. *Heredity*, 100(2), p.132.
- Géminard, C., Rulifson, E.J. and Léopold, P., 2009. Remote control of insulin secretion by fat cells in *Drosophila*. *Cell metabolism*, 10(3), pp.199-207.
- Ghosh, S.M., Testa, N.D. and Shingleton, A.W., 2013. Temperature-size rule is mediated by thermal plasticity of critical size in *Drosophila melanogaster*. *Proceedings of the Royal Society of London B: Biological Sciences*, 280(1760), p.20130174.
- Hart, J.S., 1956. Seasonal changes in insulation of the fur. *Canadian Journal of Zoology*, 34(1), pp.53-57.
- Herboso, L., Oliveira, M.M., Talamillo, A., Pérez, C., González, M., Martín, D., Sutherland, J.D., Shingleton, A.W., Mirth, C.K. and Barrio, R., 2015. Ecdysone promotes growth of imaginal discs through the regulation of Thor in *D. melanogaster*. *Scientific reports*, 5.
- Hill, R.J. and Sternberg, P.W., 1992. The gene *lin-3* encodes an inductive signal for vulval development in *C. elegans*. *Nature*, 358(6386), pp.470-476.
- Huang, M., Wheeler, D. and Fjerdingstad, E. (2013). Mating system evolution and worker caste diversity in *Pheidole* ants. *Molecular Ecology*, 22(7), pp.1998-2010.
- Ikeya, T., Galic, M., Belawat, P., Nairz, K. and Hafen, E., 2002. Nutrient-dependent expression of insulin-like peptides from neuroendocrine cells in the CNS contributes to growth regulation in *Drosophila*. *Current biology*, 12(15), pp.1293-1300.

- Kacser, H. and Burns, J.A., 1981. The molecular basis of dominance. *Genetics*, 97(3-4), pp.639-666.
- Kannan, K. and Fridell, Y.W.C., 2013. Functional implications of Drosophila insulin-like peptides in metabolism, aging, and dietary restriction. *Frontiers in physiology*, 4.
- Koyama, T. and Mirth, C.K., 2016. Growth-blocking peptides as nutrition-sensitive signals for insulin secretion and body size regulation. *PLoS biology*, 14(2), p.e1002392.
- Koyama, T., Rodrigues, M.A., Athanasiadis, A., Shingleton, A.W. and Mirth, C.K., 2014. Nutritional control of body size through FoxO-Ultraspiracle mediated ecdysone biosynthesis. *Elife*, 3, p.e03091.
- Mendes, C.C. and Mirth, C.K., 2016. Stage-specific plasticity in ovary size is regulated by insulin/insulin-like growth factor and ecdysone signaling in Drosophila. *Genetics*, 202(2), pp.703-719.
- Miner, A.L., Rosenberg, A.J. and Nijhout, H.F., 2000. Control of growth and differentiation of the wing imaginal disk of *Precis coenia* (Lepidoptera: Nymphalidae). *Journal of Insect Physiology*, 46(3), pp.251-258.
- Mirth, C.K. and Riddiford, L.M., 2007. Size assessment and growth control: how adult size is determined in insects. *Bioessays*, 29(4), pp.344-355.
- Mirth, C., Truman, J.W. and Riddiford, L.M., 2005. The role of the prothoracic gland in determining critical weight for metamorphosis in *Drosophila melanogaster*. *Current Biology*, 15(20), pp.1796-1807.
- Mirth, C.K., Truman, J.W. and Riddiford, L.M., 2009. The ecdysone receptor controls the post-critical weight switch to nutrition-independent differentiation in *Drosophila* wing imaginal discs. *Development*, 136(14), pp.2345-2353.
- Moczek, A., Sultan, S., Foster, S., Ledon-Rettig, C., Dworkin, I., Nijhout, H., Abouheif, E. and Pfennig, D. (2011). The role of developmental plasticity in evolutionary innovation. *Proceedings of the Royal Society B: Biological Sciences*, 278(1719), pp.2705-2713.
- Nässel, D.R., Liu, Y. and Luo, J., 2015. Insulin/IGF signaling and its regulation in *Drosophila*. *General and comparative endocrinology*, 221, pp.255-266.
- Nijhout, H.F., 2003. The control of body size in insects. *Developmental biology*, 261(1), pp.1-9.
- Nijhout, H.F., Riddiford, L.M., Mirth, C., Shingleton, A.W., Suzuki, Y. and Callier, V., 2014. The developmental control of size in insects. *Wiley Interdisciplinary Reviews: Developmental Biology*, 3(1), pp.113-134.
- Nijhout, H.F., Sadre-Marandi, F., Best, J. and Reed, M.C., 2017. Systems Biology of Phenotypic Robustness and Plasticity. *Integrative and Comparative Biology*, 57(2), pp.171-184.

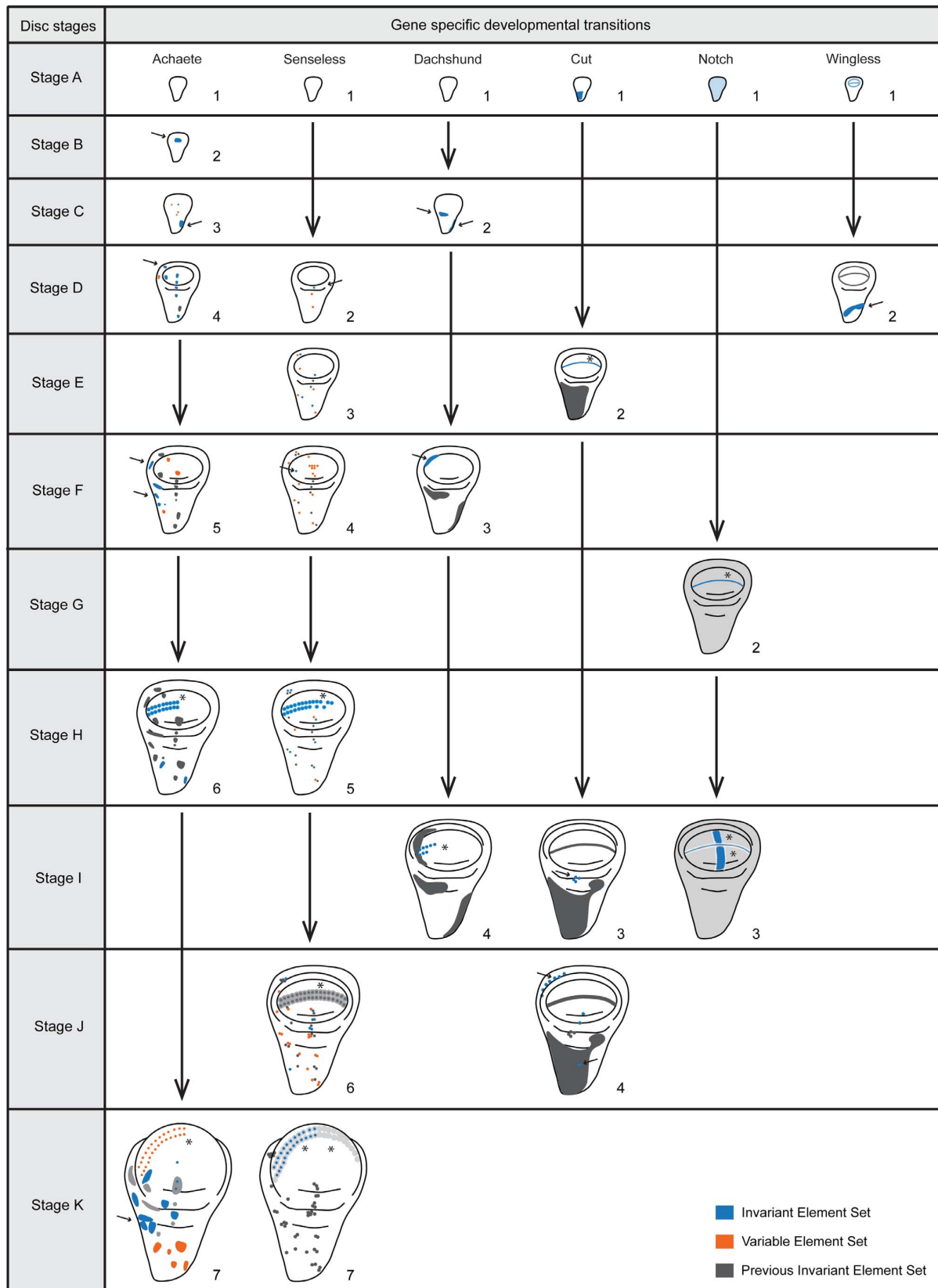
- Nijhout, H.F., Smith, W.A., Schachar, I., Subramanian, S., Tobler, A. and Grunert, L.W., 2007. The control of growth and differentiation of the wing imaginal disks of *Manduca sexta*. *Developmental biology*, 302(2), pp.569-576.
- Nolo, R., Abbott, L.A. and Bellen, H.J., 2000. Senseless, a Zn finger transcription factor, is necessary and sufficient for sensory organ development in *Drosophila*. *Cell*, 102(3), pp.349-362.
- Okamoto, N., Yamanaka, N., Yagi, Y., Nishida, Y., Kataoka, H., O'Connor, M.B. and Mizoguchi, A., 2009. A fat body-derived IGF-like peptide regulates postfeeding growth in *Drosophila*. *Developmental cell*, 17(6), pp.885-891.
- Oliveira, M.M., Shingleton, A.W. and Mirth, C.K., 2014. Coordination of wing and whole-body development at developmental milestones ensures robustness against environmental and physiological perturbations. *PLoS genetics*, 10(6), p.e1004408.
- Oyama, K., Yamada, T., Ito, D., Kondo, T. and Yoshida, K. (2015). Metal Complex Pigment Involved in the Blue Sepal Color Development of *Hydrangea*. *Journal of Agricultural and Food Chemistry*, 63(35), pp.7630-7635.
- Perrimon, N., Pitsouli, C. and Shilo, B.Z., 2012. Signaling mechanisms controlling cell fate and embryonic patterning. *Cold Spring Harbor perspectives in biology*, 4(8), p.a005975.
- Restrepo, S., Zartman, J.J. and Basler, K., 2014. Coordination of patterning and growth by the morphogen DPP. *Current Biology*, 24(6), pp.R245-R255.
- Robertson, F.W., 1963. The ecological genetics of growth in *Drosophila* 6. The genetic correlation between the duration of the larval period and body size in relation to larval diet. *Genetics Research*, 4(1), pp.74-92.
- Schubiger, M. and Truman, J.W., 2000. The RXR ortholog USP suppresses early metamorphic processes in *Drosophila* in the absence of ecdysteroids. *Development*, 127(6), pp.1151-1159.
- Schubiger, M., Carré, C., Antoniewski, C. and Truman, J.W., 2005. Ligand-dependent de-repression via EcR/USP acts as a gate to coordinate the differentiation of sensory neurons in the *Drosophila* wing. *Development*, 132(23), pp.5239-5248.
- Shingleton, A.W., 2010. The regulation of organ size in *Drosophila*: physiology, plasticity, patterning and physical force. *Organogenesis*, 6(2), pp.76-87.
- Shingleton, A.W., Das, J., Vinicius, L. and Stern, D.L., 2005. The temporal requirements for insulin signaling during development in *Drosophila*. *PLoS biology*, 3(9), p.e289.
- Shingleton, A.W., Estep, C.M., Driscoll, M.V. and Dworkin, I., 2009. Many ways to be small: different environmental regulators of size generate distinct scaling relationships in *Drosophila melanogaster*. *Proceedings of the Royal Society of London B: Biological Sciences*, 276(1667), pp.2625-2633.

- Shingleton, A.W., Mirth, C.K. and Bates, P.W., 2008. Developmental model of static allometry in holometabolous insects. *Proceedings of the Royal Society of London B: Biological Sciences*, 275(1645), pp.1875-1885.
- Slaidina, M., Delanoue, R., Gronke, S., Partridge, L. and Léopold, P., 2009. A *Drosophila* insulin-like peptide promotes growth during nonfeeding states. *Developmental cell*, 17(6), pp.874-884.
- Suster, M.L., Seugnet, L., Bate, M. and Sokolowski, M.B., 2004. Refining GAL4-driven transgene expression in *Drosophila* with a GAL80 enhancer-trap. *genesis*, 39(4), pp.240-245.
- Tang, H.Y., Smith-Caldas, M.S., Driscoll, M.V., Salhadar, S. and Shingleton, A.W., 2011. FOXO regulates organ-specific phenotypic plasticity in *Drosophila*. *PLoS genetics*, 7(11), p.e1002373.
- Teleman, A.A. and Cohen, S.M., 2000. Dpp gradient formation in the *Drosophila* wing imaginal disc. *Cell*, 103(6), pp.971-980.
- Testa, N.D., Ghosh, S.M. and Shingleton, A.W., 2013. Sex-specific weight loss mediates sexual size dimorphism in *Drosophila melanogaster*. *PloS one*, 8(3), p.e58936.
- Tobler, A. and Nijhout, H.F., 2010. A switch in the control of growth of the wing imaginal disks of *Manduca sexta*. *PLoS One*, 5(5), p.e10723.
- Wang, J. and Bell, L.R., 1994. The Sex-lethal amino terminus mediates cooperative interactions in RNA binding and is essential for splicing regulation. *Genes & Development*, 8(17), pp.2072-2085.
- Warren, J.T., Yerushalmi, Y., Shimell, M.J., O'Connor, M.B., Restifo, L.L. and Gilbert, L.I., 2006. Discrete pulses of molting hormone, 20-hydroxyecdysone, during late larval development of *Drosophila melanogaster*: Correlations with changes in gene activity. *Developmental Dynamics*, 235(2), pp.315-326.

7. Appendix

It's not about what it is, it's about what it can become.

- Dr. Seuss in *The Lorax*



Supplemental Figure 1: Patterning scheme from Oliveira et al., 2014. Differences in gene expression of six different genes (Achaete, Senseless, Daschund, Cut, Notch and Wingless) throughout development of *Drosophila melanogaster* from moult to L3 stage (Stage A) to white pre-pupae (Stage K). For the experiments, only Achaete and Senseless patterning progressions were used.